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THE SOURCE OF EXCESS CALCIUM IN HYPERCALCEMIA INDUCED BY IRRADIATED ERGOSTEROL

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In 1928 we published a communication to the effect that hypercalcemia, which comes about in normal infants or animals when irradiated ergosterol is given in great excess, has its source in the body tissues and not in the calcium of the food (1). These experiments were carried out on rats which received a ration which was almost, but not entirely, free from calcium. In order to render the trace of calcium in the diet negligible, a large amount of phosphorus was incorporated, making the Ca:P ratio about 1:500. About 2 years later another communication on the same subject was made, in which the conclusion was reached that "as stated previously we are of the opinion that the source of the marked increase in serum calcium, induced by excessive amounts of irradiated ergosterol, is the tissues, more particularly the bones which are the great storehouses of calcium in the body" (2). In the same communication it was stated that metabolism tests on dogs treated in this way showed a lack of retention of calcium, either a definite diminution or a negative balance, and that the ash of the bones of rats which had received very large amounts of irradiated ergosterol was lower than that of the control animals which had not received this addition.

In a recent paper published in this *Journal*, Jones, Rapoport, and Hodes (3) have come to the conclusion that our interpretation of the source of excess calcium is erroneous and "that the source of the excess of calcium in irradiated ergosterol hypercalcemia is the food and not the body tissue." They write as follows:

"Numerous investigators have shown that large doses of irradiated ergosterol produce a hypercalcemia. The source of this excess of serum calcium

1

has not been determined, but several workers believe that it is the body tissues especially the bones and not the ingested calcium of the food. Light, Miller, and Frey (1), Hess, Weinstock, and Rivkin (2), and Brown and Shohl (3) claim that very large amounts of vitamin D cause a decrease

TABLE I

Induction of Hypercalcemia by Means of Viosterol.   Dogs on a Calcium-Free Ration*

Dog No.	Weight	Irradiated ergosterol		Serum		Remarks
		Dose per day	No. of days given	Calcium	Phosphorus	
	kg.	*mg.*		*mg.*	*mg.*	
299	11.4	0		12.0	6.0	Complete loss of appetite. Died
	11.0	100	5	17.9		16 hrs. later. Autopsy showed
		0	1	17.7	12.0	intestinal hemorrhages
260	10.4	0		10.2	4.1	
	10.3	10	7	11.0	5.3	Appetite poor throughout re-
	9.9	10	5	10.0	5.1	mainder of experiment
	8.8	10	8	11.6	5.9	
	8.7	10	6	11.0	6.4	
	8.2	50	6	18.3	6.2	
	8.0	0	2	20.1	8.7	
		0	1	19.8	6.0	
	7.4	0	2	17.1	7.8	
		0	1	16.1	7.3	Killed. Autopsy showed slight
		0	1	16.2	7.5	hemorrhages of small intes-
		0	1	15.0	9.4	tine
		0	1	14.8	11.8	
301	9.5	0		11.0	6.8	Died during bleeding. Autopsy
	9.0	100	5	17.7	11.0	showed hemorrhages of small
		0	1	15.5	23.1	intestine and spleen

* The calcium-free ration was that recommended by Jones, Rapoport, and Hodes (3). The irradiated ergosterol was given as concentrated viosterol 10,000 D.   2.5 cc. of this solution contain 100 mg. of irradiated ergosterol (Mead Johnson and Company).

in the percentage of bone ash. . . . The most direct proof that the source of calcium is the body tissues and not the food is furnished by the work of Hess, Weinstock, and Rivkin (2, 5). They have demonstrated that it is possible to produce a vitamin D hypercalcemia on a diet low in calcium. . . . The experiments of Hess and associates are subject to criti-

cism in that the diet used may have contained sufficient calcium to produce a hypercalcemia although it was low in respect to this element."

In looking over this report it was noted that these investigators had failed to mention or to appreciate the importance, from the standpoint of calcium absorption from the intestine, of the very high ratio of phosphorus in the ration compared to that of calcium. Furthermore, their explanation was not compatible with the observation that when young rats were placed on this calcium-low ration the concentration of calcium in their serum fell from 10 to

TABLE II

Relation between Dosage of Irradiated Ergosterol and Hypercalcemia in Dogs on Varying Calcium Intake

Dog No.	Weight	Irradiated ergosterol*			Serum Ca		Ca in daily ration per kilo
		Daily dose		No. of days given	Initial	Final	
		Total	Per kilo				
	kg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
260	8	10	1.2	26	10.2	11.0	0
	8	50	6	6	11.0	18.3	0
269	17	100	6	4	11.2	17.8	27
299	11	100	9	5	12.0	17.9	0
332	10	100	10	3	11.0	14.0	21
391	9.5	100	10	3	11.8	14.9	22
441	5	50	10	9	11.9	13.4	4
441	5	50	10	10	10.5	14.4	48
301	9	100	11	5	11.0	17.7	0
119	9	100	11	8	11.2	20.5	23

* Irradiated ergosterol was given as concentrated viosterol, 10,000 or 1000 D.

6.4 mg., and that it was quickly raised to the normal merely by adding irradiated ergosterol to the ration. However, it seemed best to repeat the experiments of Jones and his coworkers, using their identical technique and feeding the dogs with the calcium-free ration which they had used and which had the following composition.

per cent

Lactose 43

Lard 22

Sucrose 33

Cane-sugar charcoal 2

Excess Ca in Hypercalcemia

Date	Weight	Irradiated ergosterol* Daily dose	No. of days given	NaHCO₃ intravenously cc.	per cent	Serum Calcium	Phosphorus	Plasma CO₂	Time of bleeding in relation to injection of NaHCO₃	Remarks
	kg.	*mg.*		*cc.*	*per cent*	*mg.*	*mg.*	*vols.*		
Mar. 24	4.8	50	8			11.9	7.0			Diet was kitchen scraps
" 26		50	2			13.4	7.0			
" 27		0	1	10	5	12.8	5.7		Before	
						10.7	5.4		60 min. after	
						10.5	6.0		4 hrs. "	
" 28	4.7					12.3			27 " "	
" 29						11.3			47 " "	
" 31						10.5	5.4			200 cc. milk added to diet
Apr. 10	4.6	50	7			12.8	5.9			
" 14		50	4			14.4	6.0			Appetite poor
" 15		0	1	10	5	14.3	6.1	36	Before	
						13.5	6.1	51	1 hr. after	
						13.8		44	4 hrs. "	
" 16		0	2	20	5	13.8		40	Before	
						13.3		53	1 hr. after	
						12.9		52	3 hrs. "	
" 24	4.5	0	8			12.2	5.1			Appetite still poor
May 1	4.4	0	7			12.0	6.4			" improving
" 8		0	7			10.5				" good
" 9		50	10			17.6	4.5			" poor

Date	*									Remarks
May 20		0	1	40	5	16.3		Before	47	May 16, 1.24 gm. Ca(H$_2$PO$_4$)$_2$·H$_2$O fed daily with diet
						15.9		1 hr. after	56	
						15.8		3 hrs. "	56	
" 21	4.6	0	2	40	10	15.3		Before	41	
						13.5		1½ hrs. after	73	
						13.4		28 " "	63	
" 22		0	1			14.8				
" 23	4.5	0	1			15.3				
" 27		0	2							
" 29		0		40	10	15.8	4.9	Before	42	
						11.5	4.1	4 hrs. after	65	
						11.6		8 " "	66	
								24 " "		
" 30	4.4	0	3			12.6	3.9			
June 4		0	5			10.3	4.6			
" 9		50	7			15.8	3.9			
" 12	4.3	0	1	40	10	14.0	4.5	Before	31	
						12.7	3.8	4 hrs. after	54	
						13.1	3.3	8 " "	45	
								48 " "		
" 14		0	2			11.4	6.0			Appetite very poor. Diet changed to meat only
" 28	4.3	0	14			14.3	6.4			
July 2		0	4			12.6	3.4			Very ill
" 7	4.1	0	5			13.0				" "
										Killed. Autopsy showed no abnormalities

* Given as concentrated viosterol, 1000 D; 12.5 cc. of this solution contain 50 mg. of irradiated ergosterol.

About 160 gm. of the above were added to the alcoholic extract of 25 gm. of wheat embryo.

When only 10 mg. of irradiated ergosterol were given daily for a short period to dogs on this ration, the calcium concentration of the serum did not rise notably (Table I). It soon became clear, however, that if this amount were increased to 50 mg. the calcium concentration could be made to rise to 18 mg. or more in 6 days (Table II). Clearly the failure of Jones and his coworkers to obtain hypercalcemia was due to the fact that irradiated ergosterol was used in insufficient amounts. With larger doses calcium is withdrawn from the tissues. It will be noted in Table II that the animals suffered from loss of appetite which is an early and characteristic symptom of hypercalcemia. Hemorrhages of the small intestine were frequently found post mortem. This has been a frequent pathological change in dogs which have suffered from hypercalcemia. The lesions are generally in the upper part of the small intestine and may also involve the stomach. These organs may likewise be the site of ulceration and blood may be found in the gastrointestinal cavity. It is also of interest that many of the dogs which have become markedly emaciated in the course of these experiments on hypercalcemia, have, nevertheless, had a surprisingly large amount of adipose tissue in the abdominal wall and within the cavity.

Table II shows that dogs have an idiosyncrasy to the development of hypercalcemia in that this reaction does not run absolutely parallel to the amount of irradiated ergosterol which they have received. This individuality has been observed likewise in infants. The same lack of conformity is true in regard to the relation of the height of hypercalcemia and intake of calcium in the diet. For example, the calcium in the serum of Dog 441 was elevated only to 13.4 mg. in spite of the fact that the animal received a daily total of 50 mg. of irradiated ergosterol; and even when the calcium intake in the ration was increased from 4 to 48 mg. daily, the largest amount given to any animal, the calcium concentration was elevated merely to 14.4 mg.

Experiments were carried out in order to ascertain whether hypercalcemia could be somewhat or entirely reduced by means of various measures. This was undertaken from a pharmacologic point of view and because it was thought that a measure of this kind

might prove to be of value in the hypercalcemia of man. Table III illustrates, in summary form, an experiment of this nature which was carried out for a period of about $3\frac{1}{2}$ months by means of intravenous injections of sodium bicarbonate. It will be seen that, with one exception, an hour or more subsequent to an injection of this kind the concentration of calcium in the serum fell and that this reduction persisted for at least 48 hours. For this purpose either 10 or 20 cc. of a 5 per cent solution of $NaHCO_3$ were injected or 40 cc. of a 10 per cent solution. The weaker solution is preferable as 10 per cent sometimes brings about edema.

TABLE IV

Calcium and Phosphorus of Organs of Dog Which Developed Hypercalcemia As the Result of Irradiated Ergosterol*

Organ	Dog 441, hypercalcemia		Dog 109, normal	
	Calcium	Phosphorus	Calcium	Phosphorus
	Per 100 gm. dry tissue		Per 100 gm. dry tissue	
	mg.	*mg.*	*mg.*	*mg.*
Lung................................	625	2158	94	448
Liver...............................	37	915	18	455
Kidney.............................	320	1203	65	728
Muscle.............................	94	769	25	584
Heart..............................	34	717	22	893
Skin................................	46	119	22	167
Brain..............................	22	524	60	1300

Bone ash, 58.7 per cent.

* A total of 1.85 gm. of irradiated ergosterol, as viosterol 1000 D, was given this dog over a period of about 3 months. See Table II. The figures for the normal heart, skin, and brain were taken from the literature.

Following these injections, the CO_2 content of the plasma rapidly rose, falling once more within a few hours. Of greater interest is the fact that the calcium in the urine and feces was definitely diminished as the result of these injections. It was natural, therefore, to suppose that the sodium bicarbonate had led to a precipitation of calcium and phosphorus into the tissues of the body. Table IV shows that this surmise was correct. It is seen that both calcium and phosphorus were found in greatly increased amounts in the lungs as well as in the kidneys of a dog in which hypercalcemia had been brought about and then reduced

by $NaHCO_3$.   Analysis of the liver showed changes in far less degree.

SUMMARY

When given in excessive amount, irradiated ergosterol induces hypercalcemia in dogs which are fed a ration absolutely free from calcium.   The failure of investigators to bring about this condition and their conclusion that the source of the excess calcium in this form of hypercalcemia is the food and not the tissues, is due to the fact that irradiated ergosterol was given in insufficient amounts.

Hypercalcemia in dogs was greatly reduced by intravenous injections of a solution of sodium bicarbonate.   Following reduction by such means, a marked excess of calcium and phosphorus was found in the lungs and the kidneys.

Addendum—Jones and Rapoport (4) have just reported that, by giving larger amounts of irradiated ergosterol, they succeeded in inducing hypercalcemia "even though there was no calcium in the diet."

BIBLIOGRAPHY

1. Hess, A. F., Weinstock, M., and Rivkin, H., *Proc. Soc. Exp. Biol. and Med.*, **26**, 199 (1928).
2. Hess, A. F., Weinstock, M., and Rivkin, H., *Proc. Soc. Exp. Biol. and Med.*, **27**, 298 (1930).
3. Jones, J. H., Rapoport, M., and Hodes, H. L., *J. Biol. Chem.*, **89**, 647
4. Jones, J. H., and Rapoport, M., *J. Biol. Chem.*, **93**, 153 (1931).

AN INVESTIGATION OF THE COMPARATIVE ASH CONTENT OF THE METAPHYSES AND SHAFTS OF BONES

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WEINSTOCK

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In an endeavor to understand more clearly the process involved in the healing of the rachitic lesion in rats when tetany is brought about by a switch from a rachitic to a normal ration (1), the percentage of ash of the metaphyses, of the shaft as well as of the entire bone, was determined.

Bone cannot be considered as a homogeneous organ. Anatomically it is differentiated and functionally it does not respond to various stimuli as a uniform organ, as may be noted in rickets when the rachitic lesion occurs in the metaphysis while the shaft remains comparatively in a normal state.

For our study the femora of young rats were used. After having been dissected from the body and freed from the associated muscle and other tissue, they were divided into three sections, two metaphyses and the shaft. The exact line of section was somewhat arbitrarily selected, but a definite area was chosen and maintained throughout the experiments. At the proximal end, the bone was cut just below the lesser trochanter while at the distal end it was cut just above the condyles. Table I shows that the ash of the metaphyses and shafts of the femora of individual rats tallied remarkably well, the variation between corresponding sides being about 1 per cent. In point of fact the error was less than this amount as, in general, the metaphyses and the shafts of two femora of each animal were pooled, thus greatly reducing differences due to sectioning of the bones and to the small amount of material available for analysis.

The two metaphyses were pooled and the shaft as well as the metaphyses was crushed and extracted with alcohol for 12 and with ether for 16 hours, after which procedure the dry weights of the samples were taken, the bones placed in a muffle, and the weights of the ash determined.. From the dry and ash weights of the metaphyses and shafts the percentage of ash of the entire bone was calculated. ' . '1 ' . , 1 ' . '.

A check on these calculations was carried out in a series in which one femur was divided into its three parts and analyzed,

TABLE I

Ash Content of Metaphyses and of Shafts of Femora of Individual Rats

Rat No.	Metaphyses, per cent ash		Difference	Shafts, per cent ash		Difference
			per cent			per cent
15329				54.8	52.9	1.9
15441	38.6	39.1	0.5	57.1	57.1	0.0
15447	22.1	23.9	1.8	51.4	51.6	0.2
15453	27.7	28.1	0.4	53.8	52.5	1.3
15454	24.8	23.3	1.5	50.0	52.4	2.4
15490	31.9	30.9	1.0	52.4	50.6	1.8
15492	35.8	36.3	0.5	53.6	54.7	1.1
15499	27.2	26.3	0.9	47.0	49.7	2.7
15612	54.3	54.3	0.0	64.1	64.2	0.1
15613	56.3	57.7	1.4	67.7	67.2	0.5
15614	55.7	57.5	1.8	65.2	65.2	0.0
15615	54.3	56.7	2.4	65.1	64.3	0.8
15616	51.6	52.7	1.1	62.3	63.5	1.2
Average..................			1.11*			1.07*

* The difference would be less where two metaphyses are pooled for examination, the procedure followed in routine analyses.

whereas the corresponding femur was analyzed as a whole. A comparison of the percentages of total ash as determined and calculated in this way is charted in Table II. The two values correspond very closely..

In our first experiment we determined the percentages of ash in the shafts, metaphyses, and whole bones of three groups of animals—normal rats which had been fed the Sherman normal diet, Diet D (2), rats which had been rendered rachitic by the

McCollum ration, Diet 3143 (3), and a third group in which
tetany had been induced in rachitic rats by a sudden switch from
the McCollum ration (Ca:P = 4:1) to the Sherman ration (Ca:P
= 1:1). In the normal group the average percentages of ash of
the metaphyses, shafts, and whole bones were 51.2, 63.8, and 56.1,
respectively. On the other hand the averages for the rachitic
group were metaphyses 34.9, shafts 53.5, and whole bones 42.0.
In the tetany group, which was on the Sherman diet for only 3
days, the averages were 36.4, 54.0, and 42.9, respectively (Table
III).

TABLE II

*Determinations of Ash of Femur Compared with Calculations Based on
Analyses of Metaphyses Plus Shaft*

Ash calculated	Ash determined	Difference
per cent	*per cent*	*per cent*
48.9	49.9	1.0
39.8	40.3	0.5
44.3	47.1	2.8
33.5	35.3	1.8
43.4	45.1	1.7
51.6	51.4	0.2
47.8	47.9	0.1
34.3	33.1	1.2
41.4	42.0	0.6
Average.............................		1.1

From the data obtained in this experiment it may be noted that
in rats rendered rachitic by this ration, osteoporosis develops in
the shaft as well as the rachitic lesion in the epiphyses; the per-
centage of ash of the shafts decreased from an average of 63.8 to
53.5. Furthermore, upon analysis of the individual figures it
will be noted that the lower the percentage of ash of the meta-
physes, the lower its percentage in the shaft; so that it would
appear that the degree of osteoporosis is dependent upon the
severity of the rachitic lesion.

When healing came about, due to a switch in diet from a rachitic
to a normal ration, the increase of ash in the metaphyses was
greater than the increase in the shaft. This reaction would appear

TABLE III

Ash Content of Metaphyses, Shafts, and Entire Femora of Normal, Rachitic, and Tetanic Rats

Weight	Diet	Radiograph Jan. 22, 1931	Radiograph Feb. 2, 1931	Serum Calcium	Serum Phosphorus	Ash, metaphyses (M)	Ash, shaft (S)	Ash, entire bone	M/S
gm.						*per cent*	*per cent*	*per cent*	
100	Sherman Diet D	Normal				51.6	65.9	56.7	0.783
122		"				52.4	64.3	57.0	0.815
100		"				49.5	62.2	54.8	0.796
100		"				52.0	63.5	56.3	0.820
112		"				49.1	63.3	54.6	0.776
80		"				49.5	61.9	54.0	0.795
100		"				51.3	64.9	56.6	0.791
82		"				50.8	61.7	55.2	0.824
94		"				53.2	65.3	58.6	0.815
92		"				51.6	64.4	56.2	0.801
100		"				52.5	64.5	56.7	0.814
Average						51.2	63.8	56.1	0.803
84	McCollum Diet 3143	Moderate rickets	No healing	10.4	4.9	40.8	56.0	48.1	0.728
70		"	"	9.4	4.7	36.3	52.9	42.2	0.686
76		"	Trace healing	} 9.5	} 5.0	34.9	53.5	42.1	0.652
84		Slight	No healing			29.0	46.8	36.4	0.619
70		Moderate	"	} 10.6	} 3.6	36.5	53.7	42.7	0.679
80		Slight	"	9.6	5.3	37.5	58.8	44.2	0.637
80		Moderate	"	} 9.8	} 3.2	41.6	58.5	48.1	0.711
80		"	"			29.7	51.2	38.1	0.581
90		"	"	} 10.0	} 4.6	35.9	55.5	43.1	0.646
84		"	"			27.0	48.4	35.1	0.558
70		"	"			34.9	53.7	42.2	0.650
Average						34.9	53.5	42.0	0.650

iet 3:43	Marked healing	8.4		44.1	58.7	49.8	0.751
21 days) followed	" "	6.5	13.9	34.2	51.9	39.9	0.658
by Sherman Diet	" "			39.4	53.9	44.8	0.731
D 3 days)	Moderate "	7.6	10.4	33.3	54.8	41.0	0.608
	" "	8.7	10.5	38.1	56.8	44.4	0.671
	Marked "	7.3	12.5	38.4	54.6	43.8	0.703
	Slight "	6.7	10.7	36.1	52.2	42.1	0.691
Sligh	" "	5.0	15.6	31.6	54.2	40.1	0.584
Mode	Moderate "			29.5	50.5	37.3	0.584
	" "	5.9	11.6	39.1	54.6	44.6	0.716
	Marked "	6.0	14.1	36.2	53.3	42.2	0.679
	" "	6.8	12.1	37.3	52.4	42.9	0.712
				36.4	54.0	42.9	0.674

20
90
90
80
00
84
70

natural inasmuch as the decrease in the ash associated with rickets was greater in the metaphyses than in the shaft. However, upon closer analysis of the figures, it was found that not only was the amount of ash laid down in the metaphyses greater, but the rate at which it was deposited exceeded that in the shaft (Table IV). This difference is probably explained by the fact that the epiphyses are more active functionally than the shaft and therefore react more quickly.

In a comparison of the ratios between the ash of the metaphyses and shaft (Table III) the values for the normal rats are very constant, averaging about 0.80. When rickets has been induced as described, this value falls to about 0.65 and is much less constant

TABLE IV

Comparison of Average Dry Weights and Ash of Metaphyses and Shaft of Femur*

Group	Metaphyses		Shaft	
	Dry weight	Ash	Dry weight	Ash
	gm.	*gm.*	*gm.*	*gm.*
A (normal).........................	0.1859	0.0954	0.1172	0.0751
B (rickets).........................	0.1103	0.0374	0.0687	0.0363
C (tetany).........................	0.1367	0.0512	0.0738	0.0398
Difference between Groups A and B.	0.0756	0.0580	0.0485	0.0388
Difference between Groups B and C.	0.0264	0.0138	0.0051	0.0035
Ratio of ash differences............		0.238		0.090

* The figures represent the combined weights of the femora of each animal.

than in the normals. In the tetany group, where healing generally had begun, the average value was but slightly higher than in the rachitic group. This reflects, though slightly, the more rapid healing in the metaphyses which is illustrated by the data in Table IV.

It is of interest to note the very small increase in ash content which can be noted by radiograph in the course of the healing of a rachitic epiphysis. Where slight healing was observed in the film, calculation indicated that only about 1 mg. of ash had been deposited. Even a lesser degree of calcification can readily be noted by the Roentgen rays.

In a second series of experiments, bone ash was determined on five groups of animals, again including a rachitic group and one in which rachitic rats had been rendered tetanic by a switch in diet; a third group consisted of rachitic rats which were parathyroidectomized and continued on the rachitic ration for 3 days following the operation; a fourth group was treated similarly to the third but given the Sherman normal diet for 3 days following operation; a fifth group of rachitic rats was treated similarly to those of the third group, in other words, continued on the rachitic ration after parathyroidectomy, but given cod liver oil for 7 days after operation. The data of this experiment are given in Table V. It will be noted that the percentages of ash of the metaphyses, of shafts, and of the entire bones were the lowest in the rachitic rats which were operated upon and continued on the rickets-producing diet (third group) although the serum calcium remained at the normal level, due to the large excess of calcium in the diet. In every other group the percentage of ash increased over that of the control rachitic group. In the rats in which tetany was induced simply by a switch in diet, the percentage of ash was higher than in the group in which tetany was brought about in a 2-fold way, in other words by parathyroidectomy plus a switch from a rachitic to a normal diet. In the latter there was practically no healing. However, the differences in these figures were not sufficiently marked to warrant an absolute conclusion.

A third series of experiments was conducted to compare the percentages of bone ash in the metaphyses, shaft, and whole bone of young rats with those of older rats, in an attempt to correlate these analyses with the concentrations of calcium and phosphorus in the serum. These rats were fed Bills' normal ration (4) for a period of 3 weeks and then killed. The percentage of ash in the older rats was higher than in the young rats and the figure representing the ratio of metaphysis to shaft ($M:S$) was also somewhat increased, indicating a relatively greater calcification of the epiphyses. On the other hand, the inorganic phosphorus of the serum of the young rats was higher than that of the older animals, showing the lack of parallelism between phosphorus of blood and ash of bone and indicating, furthermore, the need for a higher concentration of inorganic phosphorus in the blood in the growing animal.

TAB

Ash Content of Metaphyses, Shafts, and Entire Femora of Rats in Which Rickets or Tetany Had Been Induced

| Weight | Rickets,* radiograph | Subsequent ration (3 days) | Healing, radiograph | Serum | | Ash, metaphyses (M) | Ash, shaft (S) | Ash, entire bone | $\frac{M}{S}$ |
				Calcium	Phosphorus				
gm.						*per cent*	*per cent*	*per cent*	
80	Moderate	Parathyroid-ectomy	Slight	6.0	20.4	41.9	53.2	45.5	0.788
74	"		Trace			38.9	53.3	43.8	0.729
90	"	Sherman Diet D	None	5.9	12.4	37.7	52.8	43.2	0.714
94	"			6.2	21.3	41.0	54.8	46.7	0.749
Average........						39.9	53.5	44.8	0.745
110	Moderate		Moderate	4.6	10.4	39.5	53.5	44.4	0.738
100	"	Sherman Diet D	Marked	8.4	11.9	40.8	52.7	45.6	0.774
Average........						40.2	53.1	45.0	0.756
84	Moderate	Parathyroid-ectomy	Trace	11.4	5.4	34.1	52.1	39.7	0.654
90	"		"	11.7	4.8	37.1	53.9	41.8	0.688
72	"	McCollum Diet 3143	"	9.0	4.9	35.7	50.7	41.9	0.704
90	"		None	11.2	3.7	36.5	52.2	42.4	0.699
Average........						35.9	52.2	41.5	0.686

90	Moderate	Parathyroid-ectomy	McCollum Diet 3143 plus cod liver oil†	Moderate	13.0	4.8	40.2	55.7	45.7	0.722
108	"			"	10.5	5.6	39.1	53.6	44.7	0.730
78	"			Marked	11.7	7.3	39.8	53.3	44.3	0.746
104	Slight			"	12.3	6.6	42.8	56.7	47.3	0.754
Average..........					......	......	40.5	54.8	45.5	0.738
110	Slight		McCollum Diet 3143	None	12.0	4.8	37.7	52.0	43.4	0.725
74	"			"	12.6	6.0	37.7	52.1	42.7	0.724
104	Moderate			"	11.7	5.7	41.3	54.8	46.1	0.754
84	"			"	11.4	4.9	38.7	52.9	45.0	0.731
Average..........					......	......	38.8	53.0	44.3	0.734

* Rats were rendered rachitic by the McCollum Diet 3143.

† Cod liver oil was given for 7 days; the first two rats received 10 mg. daily, the latter two, 30 mg.

The Gunther-Greenberg method (5) was used throughout these experiments for estimations of inorganic phosphorus. This technique has the advantage of requiring a very small amount of serum but gives somewhat higher figures than most of the other methods commonly employed.

We have appended in Table VI some data in regard to the ash content of the epiphyses and shafts of the long bones of infants. Although these data are few, they are included as we failed to find information of the kind in the literature. The' epiphyses rather than the metaphyses were analyzed as they could be readily and sharply disarticulated from the shaft of the bone.

TABLE VI

Ash Determinations of Epiphyses and Shafts of Long Bones of Infants

Bone	Age of infant	Weight of infant	Ash, epiphyses* (E)	Ash, shaft (S)	Ash, entire bone	$\frac{E}{S}$	Remarks
		gm.	per cent	per cent	per cent		
Tibia................	28 days	3490	10.6	55.3		0.192	
Femur..............	2 mos.	2050	10.8	51.1	38.9	0.211	Atrophic
Tibia.............. }	4 "	3230	16.5	44.8	36.5	0.368	"
Fibula.............}			7.2	46.4	40.0	0.155	"

* Epiphyses disarticulated from shaft.

SUMMARY

A comparative study was made of the ash of the metaphyses and shafts of the bones of rats under normal and various pathologic conditions.

Determinations of ash showed that osteoporosis of the shaft accompanies rickets in rats and runs parallel with the loss of ash in the rachitic metaphyses.

In the course of healing not only is the absolute amount of ash deposited in the metaphyses greater than that deposited in the shaft, but calcification of the metaphyses proceeds at a more rapid rate than in the shaft.

It is possible to note by Roentgenogram the deposition of as little as about 1 mg. of ash when laid down in the rachitic epiphysis.

The bone ash of normal older rats is higher than that of younger rats, whereas the inorganic phosphorus of the serum is lower than that of the younger rats.

BIBLIOGRAPHY

1. Hess, A. F., Weinstock, M., Benjamin, H. R., and Gross, J., *J. Biol. Chem.*, **90**, 737 (1931).
2. Sherman, H. C., and Crocker, J., *J. Biol. Chem.*, **53**, 49 (1922).
3. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, **51**, 41 (1922).
4. Bills, C. E., Honeywell, E. M., and MacNair, W. A., *J. Biol. Chem.*, **76**, 251 (1928).
5. Gunther, L., and Greenberg, D. M., *J. Biol. Chem.*, **82**, 551 (1929).

ANTIUREASE*

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INTRODUCTION

It is especially desirable in the study of immunological phenomena to link closely the immune reaction with chemical reactions. This can be done if one can find a chemical method for the study of the course of the immune reaction. We thought that it might be possible to employ as antigen a substance which is both a protein and an enzyme, and to endeavor to follow antibody formation by running determinations of antienzyme activity.

Up to the present four enzymes have been reported as having been prepared in crystalline and supposedly pure condition. These are urease (1), pepsin (2), trypsin (3), and pancreatic amylase (4). But one of these (pepsin) has been reported to have been used for the production of an antienzyme (2). The results have been inconclusive. An enzyme that is to act as antigen should remain active in the blood stream; and pepsin is rapidly destroyed at the pH of mammalian blood.

We believed that crystalline urease should be especially suitable for the production of an antienzyme because normally occurring paralyzers are not present in blood; indeed, urease is protected by serum. Urease does not occur normally in blood of mammals and is extremely poisonous to them. Crystalline urease is protein

in nature, and color tests show it to contain the amino acids
necessary for antibody formation.

It is not within the scope of this paper to discuss antibodies
in general.   The mass of literature in this field is large.   Antibod-
ies have been described for something like sixteen different en-
zymes.   Some of these antibodies are normally occurring inhibi-
tory substances present in animals; others have arisen as the result
of the injection of enzymes.   While it is impossible categorically
to deny the existence of antienzymes, nevertheless it can be said
that the question is a disputed one and that past evidence is far
from convincing.   The fact that Hedin (5), by injecting rennet
into rabbits, obtained an antirennet which was 100 per cent reac-
tive to calf rennet, partially to sheep rennet, and not at all to pig
rennet, adds complications to the problem and one is faced with the
necessity of assuming either the existence of several specific
rennets, or of explaining the effect as due to a precipitation of the
rennet by antibodies generated by the foreign proteins.

There have been numerous attempts to produce an antiurease.
Moll (6) claimed to have done this by injecting dried cultures of
Micrococcus ureæ into rabbits.   He states that normal rabbit
serum possesses an inhibiting action towards this urease and that
the inhibitory effect is increased by the injections.   His results
were not uniform and some of the animals failed to react.   It is
impossible to calculate from Moll's data the activity of the urease
employed by him, for he makes no mention of having used any
buffer in his tests for urease activity, neither does he state the
temperature.   In one instance he allowed 100 mg. of his dried
enzyme to act on urea for 24 hours, causing the hydrolysis of 130
mg. of urea.   If carried out at 20° and at pH 7.0 this would indi-
cate an activity of only 1.7 of our urease units.

Carnot, Gérard, and Moissonnier (7) found soy bean urease
highly toxic to dogs and attempted to produce an antiurease by
giving gradually increasing injections.   They obtained no anti-
urease, although they obtained a precipitin which they ascribed
to the protein impurities present in their urease preparation.

Falk (8) found that urease disappeared more rapidly from the
blood of previously injected rabbits than from the blood of normal
rabbits and believed this due to a removal by the liver.

Jacoby (9) could not ascribe the disappearance of urease from

the blood of injected rabbits to the presence of an antiferment. He says: "Das Verschwinden der Urease aus dem Blutserum ist also nicht durch das Auftreten eines Antifermentes zu erklären."

EXPERIMENTAL

We first attempted to obtain antiurease in the spring of 1930, using rabbits of 4 pounds weight. The injection into an ear vein of 1 cc. of crystalline urease solution containing 100 units[1] caused convulsions after a few minutes and death within an hour. Another rabbit was given 0.5 cc. of urease intraperitoneally and still another was given 0.5 cc. subcutaneously. Both of these rabbits died within 48 hours. Still other rabbits were given 0.5 cc. of a urease solution containing 10 units per cc. intraperitoneally at biweekly intervals for 3 weeks. Blood was taken 4 days after the last injection and the serum tested for antiurease. We found that when 1 unit of urease was incubated with 1 cc. of this serum the urease activity decreased about 16 per cent, due allowance having been made for the ammonia produced from the urea of the serum. At this time we were not able to procure satisfactory jack bean meal and no more work could be done until January, 1931, when excellent jack beans were purchased from an Arkansas farmer.

The urease was obtained in the usual manner (1), but was recrystallized from 30 per cent alcohol instead of from 32 per cent acetone. The crystals gave a water-clear solution and possessed an activity of 135,000 units per gm. The urease was diluted with 0.9 per cent neutral sodium chloride solution and was injected intraperitoneally into two 5 pound rabbits. One rabbit received 2.5 units, and the other 5 units at the start. Injections were made in gradually increasing doses for 60 days. At first the injections were given every 8 days, but during the last 30 days they were given every 2 or 3 days. The final injections contained 600 urease units.

At first the reaction of the animals to the urease was followed by the precipitin test and at the end of the 1st month a definite ring was observed with a dilution of serum of 1:20. At the end of the 2nd month a test was observed at a dilution of 1:100. However,

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[1] A unit of urease has been defined as that amount which will form from urea-phosphate 1 mg. of ammonia N at 20° and at pH 7.0 in 5 minutes.

this told nothing about the presence of an antienzyme. For this reason we determined the ability of the immune serum to inhibit the power of urease to hydrolyze urea. The method as finally worked out is given below.

10 to 15 drops of blood are taken from the ear vein of the rabbit and allowed to clot normally in a 15 cc. centrifuge tube. The clot is broken up and centrifuged. 0.1 cc. of the clear serum is diluted with 1.5 cc. of 0.9 per cent neutral sodium chloride. 0.5 cc. of this solution is pipetted into a large, thick walled test-tube and to it are added 2 drops of 9.6 per cent neutral phosphate. One adds 1.0 cc. of crystalline urease (containing about 1.5 units) in 2 per cent gum arabic with constant rotation. The mixture is kept

TABLE I

Antiurease Activity of Normal and Immune Sera

Serum	Urease units				
	Rabbit 1, normal	Rabbit 2, normal	Rabbit 3, immune	Rabbit 4, immune	Rabbit 5, immune
cc.					
0	1.44	1.14	1.48	1.32	1.57
$\frac{1}{4}$	1.44	1.17	0.66	0.45	0.71
$\frac{1}{8}$	1.47	1.14	0.76	0.54	0.89
$\frac{1}{16}$	1.48	1.14	0.87	0.74	1.10
$\frac{1}{32}$	1.44	1.14	1.12	0.94	1.20
$\frac{1}{64}$	1.46	1.14	1.27	1.17	1.45

at 37° for 15 minutes, longer incubation having been found unnecessary. The tube is then brought to 20°, 1 cc. of urea-phosphate is added, and the urease activity determined in the usual manner. Controls are run in order to determine how much ammonia is produced by the action of the urease upon the serum urea and this value is subtracted from the value found for urease activity. The difference between the original activity of the urease and the activity after incubation with serum represents the antiurease activity. The quantity of antiurease that neutralizes 1 unit of urease we call 1 *antiurease unit*.

In Table I the effect of two normal sera on crystalline urease can be compared with the effect of three immune sera obtained from the rabbits mentioned above. Although different dilutions

of serum have been used, the volume has been the same in all cases. These figures show plainly that immune serum contains a substance inhibitory to urease, while normal serum does not. The effectiveness of the inhibitory substance increases relatively with dilution. When $\frac{1}{32}$ cc. of serum is used the antiurease units in sera from Rabbits 3, 4, and 5 are calculated to contain per cc. respectively 11.5, 12.2, and 11.8 antiurease units. Later, we obtained immune sera which, when tested by using $\frac{1}{32}$ cc., showed a titer of from 30 to 40 antiurease units per cc.

When normal rabbits are given injections of urease their blood urea decreases and their blood ammonia increases. We agree with the findings of Carnot, Gérard, and Moissonnier (7) and of Rigoni (10) that the cause of the symptoms and death of the animal is ammonia poisoning. Recently, Tauber and Kleiner (11), who injected crystalline urease into rabbits and mice, have supported these findings.

We have not yet determined the minimal lethal dose of urease for the rabbit, but can state that no rabbit has ever survived 60 units unless immune, or treated with antiurease. It is, of course, to be expected that the toxic action of urease will vary with the mode of introduction. It is of interest to note that after giving urease intraperitoneally we have found it in the blood stream later.

In order to obtain a blood picture of the effect produced by urease and by antiurease we have injected 50 units of urease intraperitoneally into two young, 5 pound rabbits (Flemish giants). The first rabbit was unprotected, but the second one had been given an intraperitoneal injection of 60 units of antiurease 18 hours previously. 5 cc. samples of blood were drawn from the ears into oxalate just before injecting the urease, 1 hour later, and 2 hours later. The blood was collected in tubes containing oxalate and was analyzed for ammonia. The unprotected rabbit went into convulsions after about 1 hour and showed a great increase of blood ammonia. The protected rabbit remained perfectly normal, as did its blood ammonia. Figures are given in Table II.

To determine blood ammonia we have placed 5 cc. of oxalated blood in large, thick walled tubes and have added 5 gm. of anhydrous potassium carbonate and 1 cc. of a 10 per cent solution of rosin in turpentine to prevent foaming. The ammonia was aer-

ated into graduated tubes containing 5 cc. of 0.01 N hydrochloric acid and 3 cc. of water. Caprylic alcohol was added to the receiving tube as needed, an excess being avoided. After 20 minutes aeration the ammonia was Nesslerized with 1 cc. of the Nessler solution of Folin and Wu, diluted to 10 cc., and compared with a standard Nesslerized at the same time and containing an appropriate amount of ammonia.

In order to obtain further evidence of the ability of immune serum to confer passive immunity an experiment was tried in which six normal rabbits were used. Rabbits 1 and 2 were given an injection of 90 units of antiurease 3 hours before injecting 90 units of urease. Rabbit 3 was given 90 units of antiurease just before injecting 80 units of urease. Rabbits 4 and 5 were given 90 units of urease, but received no antiurease. Rabbit 6 was given 80

TABLE II

Urease and Antiurease Effect on Blood Ammonia

	NH$_3$-N per 100 cc. blood		
	Start	After 1 hr.	After 2 hrs.
	mg.	*mg.*	*mg.*
Rabbit 1 (normal)................	0.1	4.4	5.0
" 2 (passively immunized)...	0.1	0.1	0.1

units of urease and received no antiurease. All injections were intraperitoneal except that Rabbit 3 was given the antiurease by ear vein. The results shown in Table III are clear cut and decisive.

The animals which had been protected by injections of antiurease were normal 3 days later. At this time they were given injections of 50 units of urease and showed no symptoms. The quantity of antiurease in the blood of these rabbits increased rapidly and, 15 days later, it exceeded the amount found in our rabbits which had been given sublethal injections of urease for a period of 60 days. Still later the rabbits were able to withstand 100 times the fatal dose of urease given in two injections and within 1 hour's time. It is therefore apparent that the best method of stimulating the formation of antiurease is by giving both antiurease and urease together, and in subsequent work we have always done this.

We were interested to learn whether rabbits near death from injections of urease could be restored to a normal condition by the administration of a sufficient amount of antiurease. Here six normal rabbits were used. They weighed approximately 4 pounds apiece. Each rabbit was given from 65 to 70 units of urease intraperitoneally. When the rabbit had become completely paralyzed ($1\frac{1}{2}$ to 2 hours) it was given 80 units of antiurease by ear vein. Four of the rabbits showed immediate signs of improvement and became normal within 1 hour. The other two died.

It was also of interest to see if antiurease obtained from rabbits would protect guinea pigs against urease. Accordingly two guinea pigs were given 30 antiurease units intraperitoneally. 90 minutes later the two protected guinea pigs and a third unpro-

TABLE III

Effect of Injection of Urease and Antiurease

Rabbit No.	Antiurease units	Urease units	Effect after 5 hrs.
1	90	90	Normal
2	90	90	"
3	90	80	"
4	0	90	Dead
5	0	90	"
6	0	80	"

tected were each given 15 units of urease intraperitoneally. The third guinea pig was dead 5 hours later, while the two protected animals remained normal. The serum used in this experiment had been kept on ice for 3 weeks.

SUMMARY

1. The serum of rabbits immunized with crystalline urease contains an antibody which definitely inhibits the hydrolysis of urea by urease *in vitro* as well as *in vivo*.

2. A quantitative method for estimating antiurease and expressing its activity in "antiurease units" has been devised.

3. The findings of Carnot, Gérard, and Moissonnier, of Rigoni, and of Tauber and Kleiner that the poisonous effect of injected urease is due to ammonia have been confirmed.

4. Rabbits immunized with crystalline urease have withstood 100 times the amount of urease fatal to normal animals and have shown no rise of blood ammonia.

5. The most rapid immunization in rabbits is obtained by giving intraperitoneal injections of urease-antiurease.

6. Serum from immune rabbits will confer passive immunity on normal rabbits and guinea pigs.

7. Rabbits severely poisoned by fatal doses of urease have been restored to normal by injections of antiurease.

BIBLIOGRAPHY

1. Sumner, J. B., *J. Biol. Chem.*, **69**, 435 (1926).
2. Northrop, J. H., *J. Gen. Physiol.*, **13**, 739 (1930).
3. Northrop, J. H., and Kunitz, M., *Science*, **72**, 262 (1931).
4. Caldwell, M. L., Booher, L. E., and Sherman, H. C., *Science*, **74**, 37 (1931).
5. Hedin, S. G., *Z. physiol. Chem.*, **77**, 233 (1912).
6. Moll, L., *Beitr. chem. Physiol. u. Path.*, **2**, 344 (1902).
7. Carnot, P., Gérard, P., and Moissonnier, S., *Ann. Inst. Pasteur*, **35**, 37 (1921).
8. Falk, M., *Biochem. Z.*, **59**, 316 (1914).
9. Jacoby, M., *Biochem. Z.*, **74**, 97 (1916).
10. Rigoni, M., *Arch. sc. biol.*, **14**, 203 (1929); **15**, 29 (1930).
11. Tauber, H., and Kleiner, I. S., *J. Biol. Chem.*, **92**, 177 (1931).

ON THE CARBOHYDRATES OF THE MUSCLES OF THE FROG (RANA PIPIENS)*

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In studying the carbohydrates of muscle under different conditions it is obviously very desirable to use the muscle of one leg for the experiment and the homologous muscle of the other for a control. This is possible only if the two muscles have the same carbohydrate content. Anderson and Macleod (1) have reported that in cats, rabbits, and rats homologous muscles of the opposite sides do not necessarily contain equal percentages of glycogen, though the averages of such muscles usually agree. For the frog no similar study is on record. Kato (2) has reported that the glycogen content of frogs *(Rana esculenta)* during the autumn months was about 1.24 per cent and that this amount gradually decreased in spring and early summer until it reached 0.4 to 0.2 per cent. His findings were confirmed by Bleibtreu (3). In the present paper the glycogen content of the two halves of the liver and of homologous muscles of frogs is reported and found to be so nearly identical that one can be used as a control for the other. This point having been established, the distribution of carbohydrates in normal and exercised muscle was investigated.

The sugar in tissues is believed by Cori (4) to arise from blood sugar and to depend on diffusion gradients. Power and Clawson (5), using a yeast fermentation method, found 22 to 30 mg. per cent of fermentable sugar in muscle. Cori (4), using a different technique, found a range of 20 to 40 mg. per cent, while Bischoff and Long (6) reported higher values ranging between 78 and 111

mg. per cent. However, Bischoff and Long did not remove reducing substances other than sugars from the extracts.

General Procedure

Glycogen Estimation—Winter frogs (*Rana pipiens*) purchased from Chicago were used. In preparing the muscles of frogs for glycogen determination the use of liquid air is not necessary. In Table I, it is shown that the whole muscle could be kept at room temperature for at least 2 hours without glycogen loss. The frog is first pithed, the loose skin removed, and the muscle as a whole is then separated out, immediately weighed, and dropped into the boiling potassium hydroxide. The accuracy of weighing is about ±1 mg. Glycogen was determined by the method developed in this laboratory (7) and glucose by the Folin and Wu sugar method as modified by the author (7). The glycogen conversion factor of 0.927 was used in calculating results.

Muscle Sugar Estimation—According to Somogyi (8) the addition of 1 cc. of 7 per cent copper sulfate and 1 cc. of 10 per cent sodium tungstate to 1 cc. of blood in a final dilution of 1:10 gives a protein-free filtrate very suitable for sugar determination. When these reagents were tried on tissues, the concentration of copper sulfate was not found adequate, but by increasing it to 10 per cent a clear filtrate was obtained. The technique employed is as follows:

The weighed muscle is immediately transferred to a porcelain mortar containing dry washed sand and the desired amount of 10 per cent copper sulfate. While grinding the tissue, the proper amount of 10 per cent sodium tungstate is allowed to drop from a burette or a pipette and grinding is continued until the muscle is reduced to a thin paste. The mixture is then washed into a graduated 15 cc. centrifuge tube and brought to the desired volume. The ratios of the various ingredients as used in these experiments are: to a sample of 1 gm. or less of tissue 1 cc. of 10 per cent copper sulfate, 1 cc. of 10 per cent sodium tungstate, and enough distilled water to bring the volume to the 10 cc. mark in the 15 cc. graduate. For samples weighing more than 1 gm. the same ratios were used. The mixture is allowed to stand for 15 minutes before centrifuging or filtering. The clear fluid can be decanted easily and is ready for sugar estimation.

As a means of fractionating muscle extracts ultrafiltration through a cellophane membrane, No. 600, was employed. Sahyun and Alsberg (9) have found that neither glycogen nor its first acid hydrolysis product passes through this membrane. Its permeability toward a trisaccharide (raffinose) was tested and it was found, that with the same pressure as that employed with muscle extract, a 0.45 per cent raffinose solution filters through very slowly, only 5 cc. in 15 minutes. Since McBain and Kistler (10) have found that, for a cellophane membrane No. 600 soaked in water, the diffusion rate of non-colloidal particles varies not only with the pressure but also with the size of the particle, and further that

TABLE I

Glycogen Content of Frog Muscle

The muscles of the right hind leg were removed at zero time and glycogen was determined immediately. 2 hours later the muscles of the left hind leg were removed for glycogen determination.

Muscle	Glycogen per 100 gm.							
	Frog 9		Frog 10		Frog 13		Frog 14	
	0 time Right leg	2 hrs. later Left leg	0 time Right leg	2 hrs. later Left leg	0 time Right leg	2 hrs. later Left leg	0 time Right leg	2 hrs. later Left leg
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Gracilis.............	0.760	0.760	0.900	0.870	0.850	0.850	1.020	0.975
Triceps femoris.....	0.760	0.790	0.875	0.810	0.655	0.650	0.950	0.860
Gastrocnemius......	0.920	0.975	0.845	0.780	0.775	0.775	0.927	1.000
Average..........	0.816	0.832	0.873	0.820	0.760	0.763	0.965	0.912

concentration of the solution does not influence the rate of ultrafiltration, therefore, it may be concluded that the molecule of raffinose is of such a size that it is barely able to pass through a cellophane membrane No. 600, and that a substance that passes through the membrane more rapidly probably has a smaller molecule.

EXPERIMENTAL

It is unnecessary to use liquid air or other means of preventing glycogenolysis for glycogen is not rapidly hydrolyzed in the intact frog muscle at room temperature as the following experiment shows.

The muscles of the right hind legs were removed immediately and analyzed for glycogen. The left hind leg was kept moist

<div align="center">TABLE II</div>
<div align="center">Glycogen Content of Frog Muscle</div>

Glycogen estimation was performed immediately after the removal of the muscles.

	Muscle	Left leg		Right leg	
		Weight	Glycogen per 100 gm.	Weight	Glycogen per 100 gm.
		gm.	*gm.*	*gm.*	*gm.*
Frog 2	Gracilis	1.18	1.235	0.82	1.250
	Triceps femoris	1.05	0.925	1.10	1.100
	Gastrocnemius	0.78	1.000	0.73	1.020
Average....................			1.050		1.123
Frog 3	Gracilis	0.795	1.095	0.705	1.030
	Triceps femoris	0.900	0.875	0.950	0.860
	Gastrocnemius	0.630	0.940	0.625	1.000
Average....................			0.970		0.963
Frog 4	Gracilis	0.650	1.300	0.695	1.280
	Triceps femoris	0.780	1.390	0.760	1.300
	Gastrocnemius	0.500	1.200	0.505	1.260
Average....................			1.296		1.280
Frog 5	Gracilis	0.655	0.510	0.650	0.543
	Triceps femoris	0.520	0.477	0.550	0.473
	Gastrocnemius	0.385	0.492	0.385	0.495
Average....................			0.493		0.504
Frog 6	Gracilis	0.925	1.250	0.890	1.190
	Triceps femoris	1.035	0.807	1.005	0.787
	Gastrocnemius	0.760	0.885	0.775	0.803
Average....................			0.980		0.926

with frog's Ringer's solution and allowed to stand for 2 hours at room temperature (about 18–20°). The muscles then were removed. The results are found in Table I.

By the methods described above glycogen was determined in the left and right legs of frogs. Results are found in Table II, and clearly indicate that while different muscles may have different glycogen content, homologous muscles are very similar. The gastrocnemius has the lowest glycogen content, perhaps because of its large tendon.

In the next experiment, the livers of individual frogs were divided into two parts and each was analyzed separately. There appears to be a close agreement in the glycogen content of the various lobes of the liver. The slight difference reported in Table III is probably due to experimental error, but it is so small

TABLE III

Glycogen Content of Frog Liver

Each liver was divided into two parts and each part was analyzed separately.

Frog No.	Sex	Weight of sample	Glycogen per 100 gm.	Average	Frog No.	Sex	Weight of sample	Glycogen per 100 gm.	Average
		gm.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*
5	F.	0.225	6.450		8	M.	0.202	4.150	
		0.255	6.440	6.445			0.360	3.900	4.025
6	"	0.395	9.270		9	"	0.255	3.800	
		0.410	9.360	9.315			3.750	3.720	3.760
7	"	0.200	5.550		13	"	0.235	2.310	
		0.354	5.100	5.325			0.400	2.340	2.325
10	"	0.472	6.840		14	"	0.245	5.400	
		0.520	6.860	6.850			0.210	5.100	5.250

that it can be neglected. For this experiment the frogs were selected at random, four males and four females. The livers of the female winter frogs seem to be distinctly richer in glycogen than those of the male winter frogs.

· Reducing substances are also present in practically equal amounts in homologous muscles, as is shown in Table IV. The determinations were made upon the protein-free filtrate prepared as described earlier in this paper. Furthermore, the muscles of the same leg were divided into two portions, anterior and posterior, and analyzed. The results, recorded in Table IV, show that the two legs are nearly identical with respect to glycogen content.

Table IV also presents the results of the determination of reducing substances in the protein-free muscle filtrate after hydrolysis. For these determinations 5 cc. of the filtrate were hydrolyzed in 1 N sulfuric acid for 2 hours, neutralized, made up to a definite volume, and the total reducing substances determined. The difference between the amounts found before and after hydrolysis is recorded in Table IV under the net hydrolyzable carbohydrates.

Next, 100 cc. of protein-free muscle filtrate derived from several frogs were ultrafiltered at a pressure of approximately 35 kilos

TABLE IV

Determination of Reducing Substances in Frog Muscle before and after Acid Hydrolysis

Values are expressed in terms of glucose per 100 gm. of tissue.

Frog No.	Muscle	Before hydrolysis	After hydrolysis Hydrolyzable carbohydrates, net	Total	Frog No.	Muscle	Before hydrolysis	After hydrolysis Hydrolyzable carbohydrates, net	Total
		mg.	mg.	mg.			mg.	mg.	mg.
40	Right hind leg	88	82	170	60	Right anterior	115	93	208
	Left " "	80	77	157		" posterior	118	98	216
41	Right " "	98	85	183		Left anterior	110	100	210
	Left " "	105	100	205		" posterior	115	95	210
59	Right anterior	90	95	185	76	Right hind leg	105	145	250
	" posterior	95	100	195		Left " "	107	143	250
	Left anterior	88	98	186	77	Right " "	125	220	345
	" posterior	92	98	190		Left " "	125	220	345
					78	Right " "	90	191	281
						Left " "	89	191	280

per sq.cm. The average rate of ultrafiltration was 9 cc. in 10 minutes. In Experiment A, Table V, at the cessation of ultrafiltration, the ultrafilter and cellophane membrane were washed with 25 cc. of distilled water, the washings collected in a clean flask, and designated as non-ultrafiltrable fraction. In Experiment B, Table V, 100 cc. of muscle filtrate of another series of frogs were used. Experiment B differs from Experiment A in one respect only. At the cessation of ultrafiltration 25 cc. of distilled water were introduced into the ultrafilter and ultrafiltration was resumed. This ultrafiltrate was collected separately

and termed Fraction 2. Each fraction was analyzed before and after hydrolysis. The results are found in Table V.

The data in Table V warrant the conclusion that the protein-free muscle filtrates of the frogs examined are free from glycogen as well as from the non-opalescent glycogen described by Sahyun and Alsberg (9). Indeed, it is clear that all the reducing substances, whether they reduce before hydrolysis or only after it, pass through the ultrafilter used at a fairly rapid rate. It is

TABLE V

Determination of Reducing Substances in Muscle Filtrate of Frogs before and after Ultrafiltration; Also before and after Hydrolysis of Each Fraction

Values are expressed in terms of mg. of glucose per 100 gm. of tissue.

	Before hydrolysis	After hydrolysis
	mg.	*mg.*
Experiment A		
Ultrafiltrable fraction.................	60	143
Non-ultrafiltrable fraction.............	25	42
Total of fractions...................	85	185
Experiment B		
Ultrafiltrable Fraction 1..............	85	190
" " 2..............	10	10
Non-ultrafiltrable fraction.............	00	00
Total of fractions...................	95	200

In Experiment A the average values for the filtrate before ultrafiltration were 85 mg. before hydrolysis and 183 mg. after hydrolysis. In Experiment B the corresponding values were 97 mg. and 200 mg.

therefore improbable that there is present any carbohydrate with a molecule as large as that of raffinose.

Finally, analyses similar to those made upon resting frog muscle, reported above, were made upon exercised muscle. The muscles of the right hind legs of spring frogs were removed immediately after pithing. The anterior muscles were used for glycogen estimation and the posterior for the estimation of reducing carbohydrate before and after hydrolysis of the protein-free muscle filtrate. The sciatic plexus of the left hind leg was then exposed and stimulated for 1.5 minutes with 50 tetanizing shocks per minute. As

in the control experiment, the muscles were then removed immediately for analysis. The results are shown in Table VI. It is apparent from this experiment that after exercise, there was a decrease of approximately 50 per cent in the glycogen content of the muscle, an increase of about 60 per cent in the reducing carbohydrate before acid hydrolysis of the filtrate, while the hydrolyzable carbohydrate suffered an appreciable loss in five of the six frogs used.

TABLE VI

Determination of Muscle Glycogen and of Reducing Carbohydrates before and after Acid Hydrolysis of Protein-Free Muscle Filtrate of Spring Frogs

Results are expressed in terms of glucose per 100 gm. of tissue.

Frog No.	Before exercise				After exercise			
	Glycogen	Reducing carbohydrates			Glycogen	Reducing carbohydrates		
		Before hydrolysis	After hydrolysis			Before hydrolysis	After hydrolysis	
			Hydrolyzable sugar	Total			Hydrolyzable sugar	Total
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
56	140	75	91	166	60	100	65	165
57	315	75	56	131	114	125	42	167
58	410	108	40	148	310	170	23	193
61	540	77	53	130	285	130	88	212
62	475	85	95	180	240	175	50	225
63	185	80	40	120	100	130	30	160
Average	344	83	63	145	185	138	50	187

DISCUSSION

The distribution of the glycogen in the frog requires little comment. In the first three experiments, in which winter frogs were used, muscle glycogen averaged about 0.9 per cent, while in the last experiment (Table VI), in which spring frogs were used, muscle glycogen averaged 0.34 per cent. These figures agree with the findings of earlier investigators.

The distribution of glycogen in the various muscles of the frog is quite comparable to that reported by Anderson and Macleod (1) for cats, rabbits, and rats. There is one slight difference how-

ever. Anderson and Macleod reported that the muscles of opposite sides do not necessarily contain equal percentages of glycogen. In the frog (Tables I to III) the difference is rather within the experimental error.

In determining the reducing carbohydrates of frog muscle filtrate before and after hydrolysis, no attempt was made to distinguish between the true reducing carbohydrates and reducing substances other than carbohydrates. An investigation of the true sugars of muscle is under way and so far it appears that the amount of non-sugar reducing substances is about 30 mg. per 100 gm. of tissue.

As for the source of muscle sugar, Cori's theory that it arises from blood sugar and depends upon diffusion gradients is not wholly consistent with the results obtained in the present investigation. Table VI demonstrates that muscle glycogen is responsible for a considerable portion, since after exercise there is approximately a 50 per cent decrease in muscle glycogen and a more than 60 per cent increase in the reducing carbohydrate of the muscle. The hydrolyzable carbohydrate, if it be a hexosephosphoric acid ester, appears to have suffered an appreciable loss. This last observation is in agreement with the findings of Ferdmann and Feinschmidt (11).

I wish to thank Dr. C. L. Alsberg for his interest in this investigation and for valuable suggestions and criticism.

SUMMARY

The winter frog (*Rana pipiens*) stores more muscle glycogen than the spring frog. The homologous muscles on opposite sides of the frog contain equal amounts of glycogen and the average contents of the muscles of the right and left sides are similar. The individual muscles of the same side of the frog do not necessarily contain equal percentages of glycogen. The female winter frog appears to store more glycogen than the male winter frog. After pithing, whole intact muscles of frogs do not show loss of glycogen at room temperature for at least 2 hours.

A method is described for the preparation of protein-free filtrate of muscle. Acid hydrolysis of such muscle filtrate increases its reduction power by almost 90 per cent.

Ultrafiltration through a cellophane membrane No. 600 causes all reducing and hydrolyzable carbohydrates found in the protein-free muscle filtrate of frogs to pass through the ultrafilter at one-third the rate of a solution of raffinose.

Immediately after exercise, there is a decrease in muscle glycogen, an increase in the reducing carbohydrate before hydrolysis, and an appreciable decrease in the hydrolyzable carbohydrate.

BIBLIOGRAPHY

1. Anderson, J. A., and Macleod, J. J. R., *Biochem. J.*, **24**, 1408 (1930).
2. Kato, K., *Arch. ges. Physiol.*, **132**, 545 (1910).
3. Bleibtreu, M., *Arch. ges. Physiol.*, **132**, 580 (1910).
4. Cori, C. F., *Physiol. Rev.*, **11**, 143 (1931).
5. Power, M. H., and Clawson, T. A., Jr., *J. Biol. Chem.*, **78**, p. lvi (1928).
6. Bischoff, F., and Long, M. L., *J. Physiol.*, **95**, 403 (1930).
7. Sahyun, M., *J. Biol. Chem.*, **93**, 227 (1931).
8. Somogyi, M., *J. Biol. Chem.*, **90**, 725 (1931).
9. Sahyun, M., and Alsberg, C. L., *J. Biol. Chem.*, **93**, 235 (1931).
10. McBain, J. W., and Kistler, S. S., *J. Gen. Physiol.*, **12**, 187 (1928).
11. Ferdmann, D., and Feinschmidt, O., *Z. physiol. Chem.*, **183**, 261 (1929).

STUDIES ON THE HEMICELLULOSES

II. THE COMPOSITION OF THE HEMICELLULOSE FROM COTTONSEED HULLS

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Numerous publications have appeared within recent years dealing with the hemicelluloses (1). Hudson and Harding (2) described the preparation of *l*-xylose from cottonseed hulls but did not investigate the hemicellulose. The investigation described below was undertaken to determine the composition of the hemicellulose obtained from this material.

EXPERIMENTAL

Isolation of Hemicellulose—The general method for isolating hemicelluloses from plant products is described in works on plant chemistry (3) and will not be discussed here. In this investigation 400 gm. of cottonseed hulls[1] were placed in a 6 liter flask, covered with 0.5 per cent ammonium oxalate solution, and heated in a bath of boiling water for 2 hours. The extract was decanted and the hulls were washed twice with water. The extraction with ammonium oxalate solution and washing with water were repeated twice more. This process removes the pectin and other easily soluble material. The residual hulls were mixed with 2800 cc. of 7 per cent sodium hydroxide solution and heated in a bath of boiling water for 2 hours. The mixture was allowed to cool and the solution filtered from the insoluble material. The filtrate was acidified with dilute hydrochloric acid. Then an equal volume of 95 per cent ethanol was added and the material allowed to

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[1] Cottonseed hulls obtained from a cotton oil mill as well as cottonseed hull bran obtained from the Swann Chemical Company, Birmingham, Alabama, were used. The hemicellulose appeared to be the same from both materials. The bran has no linters attached and is easier to work.

stand until the precipitated hemicellulose had settled. The hemicellulose was filtered off on a Buchner funnel, washed thoroughly with 50 per cent ethanol, and dried on a porous plate. The yield of the hemicellulose was approximately 25 per cent of the hulls used.

The hemicellulose was also prepared by extraction with cold sodium hydroxide solution but apparently the two processes gave the same material. Considerable hemicellulose remained in the hulls even after extraction a second time with a boiling solution of sodium hydroxide. This is probably because the hulls were not finely ground. Considerable hemicellulose is lost by this method of preparation because of its solubility. The filtrate from the precipitated hemicellulose is deeply colored.

The hemicellulose prepared as described above is a dark solid which is completely soluble in sodium hydroxide. Its alkaline solution is a dark pink in color. When finely ground it slowly dissolves in water.

Partial Analysis of Hemicellulose and of Cottonseed Hull Bran— When the hemicellulose was mixed with 8 times its weight of normal sulfuric acid and heated in a bath of boiling water for 15 hours, there remained 19 per cent of its weight as a dark insoluble body which we have not yet identified. For the present we will call this body X. It is at least partially soluble in sodium hydroxide solution and is reprecipitated by addition of acid. It is also partly soluble in ether to give a greenish fluorescent solution. Possibly it may not be a part of the hemicellulose molecule but merely dissolved by the alkali and precipitated by the acid along with the hemicellulose. However, at present all indications are that it is a part of the hemicellulose molecule.

Pentosan and carbon dioxide determinations were made on the hemicellulose as well as on two samples of the cottonseed hull bran. The first of these samples had been extracted by a 0.5 per cent solution of ammonium oxalate in a boiling water bath as previously described. The second sample had been extracted once by a cold 5 per cent solution of sodium hydroxide and twice by a 7 per cent solution of sodium hydroxide in a bath of boiling water. The results of these analyses are given in Table I. From these results it is possible to calculate approximately the amount of the hemicellulose in cottonseed hulls as well as the relative amounts of pentose and uronic acid.

Carbon dioxide was determined by a slight modification of the well known method of Lefèvre and Tollens (4). This method is less accurate than was formerly assumed. Certain of the uronides, especially certain of the polyuronides, are very stable and give slightly less than the theory of carbon dioxide. Numerous substances give small amounts of carbon dioxide. Apparently this is due to slight oxidation by the air. The first of these errors can be rectified in part by using 18 per cent hydrochloric acid in place of the 12 per cent acid prescribed in the regular method. When the

TABLE I

CO_2 and Pentosan Determined from Hemicellulose and from Cottonseed Hull Bran

	Pen-tosan	CO_2	l-Xylose	d-Gly-curonic acid	l-Xylose per mol d-glycuronic acid	Hemicellulose calculated from	
						CO_2	Pen-tosan
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
	per cent	per cent	per cent	per cent	mols	per cent	per cent
Hemicellulose from cottonseed hulls....	74	2.20	74	9.7	9.9		
Cottonseed hull bran extracted by 0.5 per cent ammonium oxalate*............	38.7	1.20	38.4	5.3	9.4	54.5	52.3
Cottonseed hull bran extracted by 7 per cent sodium hydroxide*................	10.1	0.33	10.1	1.45	9	15	13.7

* In a boiling water bath.

stronger acid is used, it is very essential to change the water each time in the two U-tubes where the carbon dioxide is washed. If this is not done some volatile substance passes through the wash water and is absorbed in the potassium hydroxide solution, coloring it a pale yellow. The error due to oxidation can be decreased to some extent by leading the air into the reaction flask approximately 2 inches above the boiling solution.

The per cent pentosan was determined according to the directions in "Official methods of analysis" (5). The results obtained are given in Table I. The pentose is l-xylose and the

acid is d-glycuronic acid. The weight of phloroglucide was corrected for the phloroglucide given by the d-glycuronic acid, and from the corrected weight of phloroglucide the percentage of l-xylose was calculated (6). From the percentages and molecular weights of l-xylose and d-glycuronic acid the relative number of molecules of these substances was calculated.

By dividing the per cent carbon dioxide or pentosan in the hemicellulose into 100 per cent, factors are obtained which when multiplied by the per cent carbon dioxide or pentosan in the hulls will give the percentage hemicellulose in the hulls. These factors, obtained from the data in Columns 1 and 2 of Table I, are respectively 45.4 and 1.351. If the percentage carbon dioxide, 2.35, in the hemicellulose reported by Anderson (7) is averaged in, the factor for carbon dioxide is approximately 44. This is probably more nearly correct.

The data in Column 5 indicate that there are 9 or 10 molecules of l-xylose to each' molecule of d-glycuronic acid, both in the hemicellulose and in the cottonseed hulls.

Apparently the hemicellulose molecule yields approximately 19 per cent of a substance X, 74 per cent l-xylose, and 9.7 per cent d-glycuronic acid. If the substance X is a single molecule, its molecular weight is approximately twice that of d-glycuronic acid. In that case the three substances would be present in the ratio of 10 molecules of l-xylose to 1 molecule of X and 1 molecule of d-glycuronic acid. The hemicellulose molecule would thus seem to consist of $(X)_1$ (l-xylose)$_{10}$ (d-glycuronic acid)$_1$ minus 11 molecules of water. The least molecular weight for such a body, calculated either from the per cent pentosan or carbon dioxide, would be between 1800 and 2000.

Hydrolysis of Hemicellulose—Numerous hydrolyses of the hemicellulose were carried out with amounts varying from 62 to 800 gm. In all of these the hemicellulose was mixed with 8 times its weight of 4.5 per cent sulfuric acid and heated in a bath of boiling water for 17 hours. The solution was filtered from the insoluble material. The filtrate was neutralized with barium carbonate. The barium sulfate was filtered off and the filtrate concentrated *in vacuo* to a small volume. The barium salt of the aldobionic acid was precipitated by addition of a large volume of ethanol. The salt was triturated in ethanol until it became

granular, then it was filtered from the sugar solution. The filtrate was concentrated to a small volume. The gum was dissolved in glacial acetic acid and the solution seeded with *l*-xylose. After standing in the refrigerator the solution became solid with crystals. These were filtered off and identified as *l*-xylose by their melting point and optical rotation. The filtrate was again concentrated and a second crop of *l*-xylose was obtained.

The yield of the various substances obtained, calculated from the weight of hemicellulose used, was approximately as follows: 19 per cent as the insoluble substance *X*, 10 per cent as the barium salt of the aldobionic acid, and from 50 to 60 per cent as *l*-xylose. The non-crystalline syrups varied considerably in amount. They consist chiefly of *l*-xylose which is kept from crystallizing by small amounts of impurities.

Barium Salt of the Aldobionic Acid—The barium salt of the aldobionic acid obtained as described above is usually quite dark in color. It always contains some iron and if the hemicellulose is not washed thoroughly after acidification the salt will contain large amounts of sodium. This latter can be removed by dissolving the salt in a small volume of water, adding excess sulfuric acid and a large volume of ethanol to precipitate the sodium sulfate. The free acid can then be converted back to the barium salt as already described.

The iron is difficult to remove. It apparently is not present as a salt of the carboxyl group. It can be removed by dissolving the barium salt in water, adding barium carbonate or a small volume of barium hydroxide, and passing hydrogen sulfide through the solution. Decolorizing carbon is then added and the precipitate filtered off. The filtrate is acidified with sulfuric acid and some of the water distilled off *in vacuo*. This removes the hydrogen sulfide. The acid is again converted to the barium salt by treatment with barium carbonate. Very often this barium salt will have a higher per cent barium and a lower per cent carbon dioxide than the theory. The solution of the barium salt must not be heated with barium hydroxide.

Several lots of barium salts were prepared at different times as previously described. These were separated into fractions by solution in water and addition of varying amounts of ethanol. Analysis of the salts indicated that they contained an aldobionic

acid consisting of 1 molecule of a hexoseuronic acid combined with 1 molecule of either a pentose, methyl pentose, or hexose.   However, it was difficult to decide from the analytical data which class of sugar was present.   This point was cleared up by identifying the acid as d-glycuronic acid and the sugar as l-xylose as described later.

In one case 90 gm. of crude barium salts were dissolved in 500 cc. of water and the solution filtered.   An equal volume of 95 per cent enthanol was added.   This precipitated 35 gm. of dark salt (A).   This salt was purified by treatment with hydrogen sulfide.   On analysis it gave 11.1 per cent carbon dioxide, 24.0 per cent barium, and showed $[\alpha]_D^{25} = +52°$.   The barium salt of pentose aldobionic acid would give 11.18 per cent carbon dioxide and 17.45 per cent barium.

The filtrate from Salt A was diluted with half its volume of ethanol.   This precipitated a small amount of salt which was discarded.   The filtrate was diluted with a very large volume of ethanol.   This precipitated 33 gm. of barium salt (C).   Without further purification this salt was analyzed.   It gave 10.8 per cent carbon dioxide, 17.23 per cent barium, 30 per cent l-xylose, and in 4 per cent solution showed $[\alpha]_D^{25} = +63°$.   It was evidently the barium salt of a pentose aldobionic acid consisting of 1 molecule of d-glycuronic acid combined with 1 molecule of l-xylose with the loss of 1 molecule of water.   While the structure of this acid has not yet been studied further, it undoubtedly is similar to other acids of this series described by previous investigators (8).

Identification of Components of the Aldobionic Acid—The naphthoresorcinol (9) test and the yield of carbon dioxide by the method of Lefèvre and Tollens proved the presence of a hexoseuronic acid in the aldobionic acid.   Careful oxidation of the barium salt of the aldobionic acid with nitric acid gave no mucic acid.   This proved the absence of d-galactose and d-galacturonic acid.   The high percentage of pentosan given by the barium salt, after correction for the furfural phloroglucide given by the uronic acid, proved the presence of a pentose or methyl pentose.

Identification of the l-Xylose—Barium Salts A and C described above were mixed and 37 gm. of the mixture were dissolved in 250 cc. of 4 per cent sulfuric acid.   An additional 5.5 gm. of sulfuric acid was added to precipitate the barium.   This solution was heated in the autoclave under a gauge pressure of 14 pounds for

7 hours. The solution was neutralized with barium carbonate and filtered. The filtrate was concentrated under reduced pressure to a small volume. A large volume of alcohol was added to precipitate the barium salts. After the gummy salts had settled, the sugar solution was filtered and concentrated under reduced pressure to a syrup. This was dissolved in glacial acetic acid and seeded first with *l*-rhamnose and later with *l*-xylose. No crystals formed. A large volume of alcohol was again added. This precipitated a small amount of gummy material.[2] This was filtered off and the filtrate again concentrated to a syrup. This was dissolved in glacial acetic acid and seeded with *l*-rhamnose. No crystals formed. It was then seeded with *l*-xylose and soon became solid with crystals. These were filtered off and dissolved in a small volume of water. The solution was clarified by decolorizing carbon and the filtrate diluted with 5 times its volume of glacial acetic acid and seeded with *l*-xylose. After standing in the refrigerator, the crystals were filtered off and identified as *l*-xylose. They melted at 145° and there was no change in the melting point when they were mixed with pure *l*-xylose. In 4 per cent solution they showed $[\alpha]_D = +19.4°$ while a sample of pure *l*-xylose under the same conditions showed $[\alpha]_D = +19°$.

Identification of d-Glycuronic Acid—The barium salts obtained by hydrolysis of the aldobionic acid in the autoclave were used to identify the uronic acid. They were dissolved in water and freed of barium by careful addition of sulfuric acid. Then 60 cc. of nitric acid, density 1.15, were added and the solution concentrated in an evaporating dish on a bath of boiling water to a volume of 200 cc. The solution was filtered from barium sulfate and the filtrate concentrated to a syrup. This was placed in the refrigerator overnight. No mucic acid appeared but a small amount of oxalic acid crystallized out. This was filtered off, dried, and found to melt at 100°. The syrup was dissolved in water and again evaporated down. It was then dissolved in 150 cc. of water, an excess of calcium carbonate added, and the solution heated in a bath of boiling water.[3] The hot solution was filtered from calcium oxalate

[2] Small amounts of impurities hinder greatly the crystallization of sugars.

[3] The potassium acid saccharate test for *d*-glucose and *d*-glycuronic acid is less reliable in the presence of oxalic acid. This can be removed as indicated above.

and calcium carbonate. The filtrate was treated with a slight excess of potassium carbonate to remove the calcium and the calcium carbonate filtered off. The filtrate was acidified with acetic acid, concentrated to a small volume on a bath of boiling water, and set in the refrigerator. A large amount of potassium acid saccharate crystallized out. When this was recrystallized from hot water, the crystals appeared identical with crystals of known potassium acid saccharate. In water they gave an acid solution. They were converted into the silver salt ((6) p. 101). This salt on analysis gave 50.97 per cent silver. The theory for silver saccharate is 50.86 per cent silver. Evidently d-glycuronic acid is present in the aldobionic acid.

DISCUSSION

While the exact structure of the hemicellulose is largely un-known, the d-glycuronic acid and the body X probably occupy extreme positions in the molecule, while the xylan makes up the mid-portion of the molecule.

The ease of hydrolysis of the hemicellulose would indicate that its molecule is held together by glucosidic unions. On the other hand, the hemicellulose is in all probability joined to the cellulose of the cottonseed hulls by an ester (10) union between its carboxyl group and a hydroxyl group of the cellulose.

Some of the plant gums and some other hemicelluloses are known to give rise on hydrolysis to insoluble products. It is quite possible, therefore, that the general structure indicated above is rather common among these substances. Gummosis in plants might often be due to hydrolysis of the ester union, with or without further change of the molecule. Furthermore, if the body X is like the substances found in the natural glucosides, these substances might often originate through rupture of a glucosidic union in the sugar portion of the hemicellulose molecule.

The early methods for preparing l-xylose (2) consisted in first isolating the hemicellulose and then hydrolyzing this substance. In general the pentoses are in the water- and alkali-soluble portions of the plant. In many cases the pentose is undoubtedly held to the plant body through a hexoseuronic acid.

It appears that cottonseed hulls offer a very promising source of d-glycuronic acid.

SUMMARY

A hemicellulose has been isolated from cottonseed hulls and its composition studied. It contains d-glycuronic acid, l-xylose, and another body. Among the products of hydrolysis an aldobionic acid has been isolated and its composition determined. A partial structure has been suggested for the hemicellulose and the possible relation of the hemicellulose to certain other plant products has been discussed.

This work is being continued as rapidly as possible.

BIBLIOGRAPHY

1. O'Dwyer, M. H., *Biochem. J.*, **17**, 502 (1923); **20**, 656 (1926).   Norman, A. G., *Biochem. J.*, **23**, 1353 (1929).   Norris, F. W., and Preece, I. A., *Biochem. J.*, **24**, 59 (1930).   Hagglund, E., Klingstradt, F. W., Rosenquist, T., and Urban, H., *Z. physiol. Chem.*, **177**, 249 (1928).
2. Hudson, C. S., and Harding, T. S., *J. Am. Chem. Soc.*, **39**, 1038 (1917).
3. Schorger, A. W., The chemistry of cellulose and wood, New York, 153 (1926).
4. Lefèvre, K. U., and Tollens, B., *Ber. chem. Ges.*, **40**, 4513 (1907).
5. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 2nd edition, 120 (1925).
6. van der Haar, A. W., Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin, 71 (1920).
7. Anderson, E., *J. Biol. Chem.*, **91**, 559 (1931).
8. Anderson, E., and Crowder, J. A., *J. Am. Chem. Soc.*, **52**, 3711 (1930).   Anderson, E., and Otis, L., *J. Am. Chem. Soc.*, **52**, 4461 (1930).   Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, **74**, 613 (1927).
9. Tollens, B., *Ber. chem. Ges.*, **41**, 1788 (1908).
10. Schmidt, E., Meinel, K., and Zintl, E., *Ber. chem. Ges.*, **60**, 503 (1927).

THE INFLUENCE OF FEEDING PROTEINS, AMINO ACIDS, AND RELATED SUBSTANCES UPON CREATINE-CREATININE METABOLISM

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INTRODUCTION

The literature of biochemistry from 1905 until the present contains evidence to show that creatine is a product of the endogenous metabolism and that it is probably used in the formation of anabolic products in the organism. According to Hunter (1), "Its rate of production is in all likelihood regulated by an internal demand, and it is not to be expected that it should be accelerated by an excessive supply of precursors any more than the production of adrenaline or thyroxine would be increased by the administration of a dose of tyrosine."

There are at least two known facts that would seem to favor this view. The creatine concentration of the muscles of a given species

of animal and the creatinine elimination in the urine of man on a meat-free diet are usually constant. There is also a mass of negative evidence for the amino acids as possible precursors of creatine in the animal body. However, many of these small scale failures to obtain an increase in muscle creatine, or in creatine and creatinine elimination in the urine, may have been due to an unsuccessful attempt to reproduce conditions favorable to their formation. Other failures might have been due to the fact that an investigator believed that creatine formation in the body was a slow process, and, after feeding small amounts of amino acids for a few weeks, would usually obtain negative results. On the other hand a positive demonstration of the origin of creatine from protein and amino acid precursors is definite and convincing and should not be discounted by negative experiments of doubtful value.

Recent evidence would seem to show that our ideas of creatine and creatinine metabolism are undergoing rapid changes. If it can be definitely shown that these substances take origin from some precursors in the diet, then the idea that they are entirely of endogenous origin is no longer tenable. Some years ago cystine was shown by Harding and Young (2) and by Gross and Steenbock (3) to give increases in creatine excretion. Recent work by Bergmann and Zervas (4), Bergmann and Köster (5), Brand and coworkers (6), and Abderhalden and Buadze (7) would seem to show that arginine, glycine, histidine, and purines must be considered as precursors of creatine and creatinine. Thus at 'the present time there is definite and convincing evidence that these substances may also have an exogenous origin from amino acids of the diet.

Several investigators who have studied the origin of creatine in the body have held the view that a single amino acid, *e.g.* glycine or arginine, would prove to be the specific precursor in this connection. Very few experiments have been carried out with other amino acids. Both Thomas (8) and Hunter (1) believe that free arginine is not the mother substance of creatine but the latter investigator admits the possibility that the arginine combined in the protein molecule may be a source of this substance. On the other hand Mitchell and Hamilton (9) believe that the evidence for free arginine is very convincing.

About 3 or 4 years ago, at the suggestion of Professor Myers, experiments were carried out in this laboratory on young rats and mice, in which it was found that d-arginine monohydrochloride caused slightly larger increases in muscle creatine than creatine itself when each of these substances formed 5 per cent of the standard casein diet, thus furnishing further evidence that this amino acid may be a possible creatine precursor. With the positive evidence for a few other amino acids at that time it was decided to make a systematic study of the effect of proteins, amino acids, and related substances upon creatine formation in the muscles of young rats, and on creatinine elimination in the urine of both the rat and man, the results of which are given in this paper.

Creatine Formation in Muscles of Rat

Young rats were fed on Sherman's Diet B[1] for a period of 10 days after weaning. They were all in good condition and weighed between 40 and 55 gm. Amounts of purified amino acids and other related substances up to 1 gm. were either fed as such or mixed with a small amount of the above diet. The ration was removed until practically complete consumption of the amino acid was obtained (usually 4 to 6 hours) and then replaced in the cage. At the end of 17, 24, 36, or 48 hours the animals were killed by a blow on the back of the head and muscle tissue was removed from the hind legs, cut up fine with scissors, and analyzed for creatine (total creatinine) by the method of Rose, Helmer, and Chanutin (10). All samples were under the acid within a few minutes after the death of the animal. Many of the colorimetric comparisons of creatinine were made by three different investigators with comparable checks in most cases.[2]

The source of the supplements used in these studies was as follows: d-arginine monohydrochloride was prepared from gelatin by the method of Cox (11); histidine, free from cystine, by the method of Vickery and Leavenworth (12); glycine, Kahlbaum; dl-phenylalanine and l-tyrosine, Special Chemicals Company;

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[1] Whole wheat flour two-thirds, whole milk powder one-third, and $CaCO_3$ and NaCl, each, 1 per cent of the weight of the wheat.

[2] The kidneys of many of these rats were examined by Dr. Robert A. Moore of the Institute of Pathology, who was unable to find any pathological changes resulting from the amino acid feeding.

d-glutamic and *l*-aspartic acids, *dl*-valine, *l*-leucine, *l*-cystine, *dl*-alanine, choline hydrochloride, sarcosine, and the synthetic amino fatty acids were all Eastman products. Taurine was prepared from ox bile.

In Table I is given the average muscle creatine concentration of 118 young rats, most of which served as litter mate controls for the experimental animals. An average value of 4.0 ±0.02 mg. per gm. of muscle was obtained, which corresponds very closely to the average value of 0.42 per cent obtained by Chanutin and Silvette (13) under similar conditions.

In Table II will be found the results obtained with 95 animals on various amino acids, eight on creatine, three on glycocyamine, and six each on casein and edestin. The average per cent in-

TABLE I

Creatine Content of Rat Muscle

Values for 118 animals.

	Age	Body weight	Creatine content per gm. muscle
	days	*gm.*	*mg.*
Minimum.	36	40	3.29
Maximum.	41	57	4.64
Average.	38	48	4.00 ±0.02

creases in muscle creatine over the control value, from lowest to highest, were as follows: alanine 12.5, glycine 15.4, aspartic acid 17.0, glutamic acid 20.5, phenylalanine 21.8, tyrosine 22.3, leucine 22.3, choline hydrochloride 23.3, creatine 23.6, histidine 24.0, arginine monohydrochloride 26.8, casein 31.2, edestin 31.2, valine 34.3, cystine 37.0, and glycocyamine 48.5.[3] Variations in in-

[3] In order fully to convince ourselves that these increases were really due to creatine, determined as creatinine, the following experiment was carried out. The muscle creatine was determined by precipitating the creatinine by the potassium picrate method of Baumann and Ingvaldsen (14). 5 gm. of combined muscle tissue from three young control rats gave 2.98 mg. of creatine per gm. of muscle, while a similar amount from three young rats, each receiving 1 gm. of glycine for 24 hours, gave 3.37 mg. per gm., an increase of 13.1 per cent. This amount compares favorably with the average increase of 15.4 per cent obtained for this amino acid by the method of Rose, Helmer, and Chanutin. Thus it would seem that the increases observed in these studies were not due to any other chromogenic substances in the muscle filtrates, and are therefore due to creatinine alone. See Hunter

TABLE II

Influence of Feeding Proteins, Amino Acids, and Related Substances upon Creatine Formation in Rat Muscle

Average control muscle creatine was 4 mg. per gm.

Substance fed	Muscle creatine	Increase	Substance fed	Muscle creatine	Increase
Glycine			dl-Valine		
gm.	mg. per gm.	per cent	gm.	mg. per gm.	per cent
0.75	4.72	18.0	1.00	5.57	39.3
1.00	5.06	26.5	0.50	4.96	24.0
1.00	4.87	21.7	0.50	4.64	16.0
1.00	4.78	19.5	1.00	5.42	35.5
1.00	4.12	3.0	1.00	6.31	57.8
0.75	4.50	12.5	1.00	5.33	33.3
0.50	4.64	16.0			
0.50	4.23	5.8	Average..	5.37 ±0.4	34.3
1.00	4.64	16.0	d-Glutamic acid		
Average...	4.62 ±0.03	15.4	1.00	4.69	17.3
			1.00	5.15	28.8
dl-Alanine			0.50	4.64	16.0
0.50	4.18	4.5	0.50 ·	4.50	12.5
0.50	4.27	6.8	1.00	4.77	19.3
1.00	4.43	10.8	1.00	4.96	24.0
1.00	4.90	22.5	1.00	4.96	24.0
1.00	4.43	10.8	1.00	4.96	24.0
1.00	5.06	26.5			
0.75	4.35	8.8	Average..	4.82 ±0.05	20.5
1.00	4.96	24.0	l-Aspartic acid		
1.00	4.37	9.3			
1.00	4.51	12.8	1.00	4.64	16.0
1.00	4.64	16.0	1.00	4.64	16.0
1.00	4.47	11.8	1.00	4.77	19.3
1.00	4.45	11.3	1.00	4.06	1.5
1.00	4.45	11.3	1.00	4.64	16.0
1.00	4.55	13.8	1.00	4.64	16.0
1.00	4.14	3.5	1.00	4.77	19.3
1.00	4.35	8.8	1.00	4.96	24.0
			1.00	5.06	26.5
Average...	4.50 ±0.04	12.5	1.00	4.64	16.0
			Average..	4.68 ±0.06	17.0

TABLE II—*Continued*

Substance fed	Muscle creatine	Increase	Substance fed	Muscle creatine	Increase
l-Cystine			*d*-Arginine HCl		
gm.	*mg. per gm.*	*per cent*	*gm.*	*mg. per gm.*	*per cent*
1.00	5.41	35.3	1.00	4.64	16.0
1.00	4.20	5.0	0.50	4.60	15.0
1.00	5.80	45.0	0.50	4.64	16.0
1.00	6.31	57.8	1.00	4.96	24.0
1.00	5.81	45.0	1.00	5.33	33.3
1.00	5.15	28.8	1.00	5.33	33.3
1.00	6.07	51.8	1.00	5.06	26.5
1.00	5.67	41.8	1.00	5.00	25.0
1.00	5.42	35.5	1.00	5.33	33.3
1.00	4.96	24.0	1.00	5.33	33.3
			1.00	5.57	39.3
Average...	5.48 ±0.04	37.0			
			Average..	5.07 ±0.07	26.8
Histidine			*l*-Leucine		
1.00	4.77	19.3			
1.00	4.96	24.0	1.00	4.96	24.0
1.00	5.15	28.8	1.00	4.96	24.0
1.00	4.96	24.0	1.00	4.82	20.5
			1.00	4.82	20.5
Average...	4.96 ±0.05	24.0			
			Average..	4.91 ±0.02	22.3
dl-Phenylalanine			Choline HCl		
1.00	4.57	14.3			
1.00	4.63	15.8	1.00	4.64	16.0
1.00	4.77	19.3	1.00	4.96	24.0
0.50	4.60	15.0	1.00	5.80	45.0
0.50	5.80	45.0	1.00	4.64	16.0
			1.00	4.77	19.3
Average...	4.87 ±0.14	21.8	1.00	4.77	19.3
l-Tyrosine			Average..	4.93 ±0.06	23.3
1.00	4.15	3.8	Glycocyamine		
1.00	4.77	19.3			
1.00	4.43	10.8	1.00	5.80	45.0
1.00	5.20	30.0	1.00	6.22	55.5
0.50	4.68	17.0	1.00	5.80	45.0
0.50	4.60	15.0			
1.00	4.96	24.0	Average..	5.94 ±0.08	48.5
1.00	5.26	31.5			
1.00	5.55	38.8			
1.00	5.26	31.5			
Average...	4.89 ±0.09	22.3			

TABLE II—*Concluded*

Substance fed	Muscle creatine	Increase	Substance fed	Muscle creatine	Increase
Creatine			Casein		
gm.	*mg. per gm.*	*per cent*	*gm.*	*mg. per gm.*	*per cent*
1.00	5.20	30.0	4.5	5.80	45.0
1.00	4.89	22.3	3.5	5.80	45.0
0.50	4.60	15.0	5.0	5.05	26.2
0.50	4.60	15.0	8.5	5.15	28.7
1.00	4.96	24.0	8.5	5.05	26.2
1.00	4.96	24.0	8.0	4.64	16.0
1.00	5.15	28.8			
1.00	5.21	30.2	Average..	5.24 ±0.11	31.2
Average...	4.95 ±0.05	23.6			
Edestin					
9.1	4.96	24.0			
8.7	4.96	24.0			
9.4	5.80	45.0			
13.0	5.33	33.3			
11.0	4.64	16.0			
12.2	5.84	46.0			
Average...	5.25 ±0.12	31.2			

creases from 5 to 30 per cent were usually obtained for the same amino acid. This was probably to be expected, since it is well known that one may find large variations in muscle creatine under different experimental conditions.

In Table III will be found data for the per cent conversion of amino acid nitrogen into creatine nitrogen, from lowest to highest, as follows: alanine 1.5, glycine 1.9, histidine 1.8, arginine mono-hydrochloride 2.0, aspartic acid 3.2, leucine 5.0, glutamic acid 6.0, tyrosine 6.9, cystine 7.0, phenylalanine 7.3, and valine 8.6.

From the results obtained it is clear that *all amino acids of the protein molecule studied have the power of increasing the normal creatine content of young rat muscle* under the conditions of these studies. The process is also fairly rapid, reaching its maximum within 17 to 24 hours after ingestion of the supplement. The feeding of 1 gm. quantities of recrystallized creatine gave some-what smaller increases than did some of the amino acids, while

glycocyamine, as is well known, gave large increases over the control level. Since amino acids *per se* are capable of raising muscle creatine, it is very likely that protein feeding would do the same under similar experimental conditions. Such was found to be the case with both casein and edestin. 1 gm. quantities of choline hydrochloride gave increases similar to those of the amino

TABLE III

Showing Conversion of Amino Acid Nitrogen into Creatine Nitrogen

Average amount of amino acid fed to group		Equivalent amount of N fed to group	Average increase in creatine per gm. muscle	Equivalent increase in creatine N per gm. muscle	Average body weight of group	Average equivalent weight of muscles of group*	Equivalent increase in creatine N in muscle mass of body	N of amino acids transformed into creatine N
	mg.	*mg.*	*mg*	*mg.*	*gm.*	*gm.*	*mg.*	*per cent*
Glycine	833.0	155.3	0.60	0.20	46.6	15.2	3.0	1 9
Alanine	991.0	155.5	0.50	0.16	46.6	15.2	2.4	1.5
Valine	833.0	99.6	1.37	0.48	55.0	18.0	8.6	8.6
Glutamic acid	875.0	83.3	0.82	0.29	51.5	16.8	5 0	6.0
Aspartic acid	1000.0	105.2	0.68	0.22	47.4	15.5	3.4	3.2
Cystine	1000.0	116.6	1.48	0.51	49.2	16.1	8.2	7.0
Histidine	1000.0	270.9	0.96	0.31	50.0	16.4	5.1	1.8
Phenylalanine	800.0	67.8	0.87	0.28	53.1	17.4	4.9	7.3
Tyrosine	900.0	69.6	0.89	0.29	50.4	16.5	4.8	6.9
Arginine HCl	909.0	241.8	1.07	0.34	44.4	14.5	4.9	2.0
Leucine	1000.0	106.7	0.91	0.29	56.0	18.2	5.3	5.0

* Based upon the figures of Jackson and Lowrey (15) showing that 1 gm. of body weight of a 42 day old rat will contain 0.327 gm. of muscle tissue.

acids, while negative results were obtained with a small number of animals on sarcosine, nucleic acid, betaine hydrochloride, and taurine.

Next it was desired to determine whether amino fatty acids not occurring in the protein molecule could give increases comparable to those given by the amino acids above. However, 1 or 1.5 gm. quantities of α-aminoisobutyric, *dl*-α-amino-*n*-butyric, *dl*-α-amino-

α-methylbutyric, α-aminocaproic, *dl*-α-aminocaprylic, and *dl*-α-aminovaleric acids, were not found to give increases in muscle creatine above the normal average in these experiments.

Creatinine Elimination in Urine of Rat

It is generally accepted at the present time that a diet rich in protein may cause creatinuria. The experimental subjects in most cases have been young animals, women or children, or patients suffering from muscular dystrophies. For this reason it is not generally believed that high protein feeding serves to

TABLE IV

Specific Dynamic Effects of Amino Acids Compared with Their Ability to Form Muscle Creatine

Amino acid	Specific dynamic effect Relative effect per gm. ingested (glycine = 100)	Muscle creatine Average increase per gm. muscle over controls (glycine = 100)
	per cent	per cent
Glycine	100	100
Alanine	69	81
Valine	None	222
Leucine	35	145
Phenylalanine	135	142
Tyrosine	72	144
Cystine	28	241
Glutamic acid	38	135
Aspartic "	25	111
Arginine	25–30	174
Histidine	None	156

provide an exogenous origin of creatine in the adult. Since we have used young rats in our experiments, it was decided to confirm the above results, if possible, by using the creatinine excretion of the adult rat as an index of the process. The results obtained are presented below.

Pied rats weighing between 200 and 300 gm. were used. Each animal was placed in a separate round, wire cage which had a china plate for a base, with a wire screen of proper mesh to prevent the passage of food and feces. Tap water and Sherman's Diet B were fed *ad libitum*. A little distilled water containing 2 drops

TABLE V
Effect of Feeding Amino Acids upon Creatinine Excretion in the Rat

Column group 1

2 day control period	2 day experimental period	Increase
Glycine, 1.0 gm.		
mg.	mg.	per cent
9.7	12.8	32.0
10.8	14.0	30.0
9.7	15.0	54.6
11.5	15.0	30.4
8.5	14.3	68.2
11.5	12.5	9.0
14.6	18.8	28.7
11.7	13.8	18.0
11.2	15.0	33.9
17.9	21.7	21.2
20.0	25.3	26.5
13.8	25.0	81.8
15.0	25.0	66.6
17.8	22.0	23.6
19.0	25.7	35.3
16.5	21.0	27.2
12.7	16.5	29.9
13.2	20.0	51.5
26.5	31.6	19.2
17.9	22.1	23.4
16.4	23.5	43.3
Average.......		35.9
l-Aspartic acid, 1.0 gm.		
10.8	11.9	10.1
13.0	13.3	
10.1	7.0	
10.1	12.0	18.8
13.8	14.8	7.2
10.8	12.1	12.0
15.0	18.2	21.3
19.0	20.0	5.2
13.6	16.6	22.0
15.3	22.5	47.1
17.8	20.0	12.3
Average.......		14.2

Column group 2

2 day control period	2 day experimental period	Increase
d-Arginine HCl, 1.0 gm.		
mg.	mg.	per cent
13.1	16.1	22.9
16.1	18.1	12.4
11.5	14.5	26.5
12.8	16.4	28.1
14.7	17.3	17.6
15.1	19.1	26.5
13.0	20.0	53.8
Average.......		26.8
dl-Alanine, 1.5 gm.		
12.6	20.0	58.7
13.1	20.8	58.8
16.6	13.5	
17.6	22.8	29.5
19.3	24.7	28.0
16.6	17.3	4.2
15.0	20.0	33.3
17.1	19.1	11.6
Average.......		28.0
dl-Valine, 1.0 gm.		
17.2	19.8	15.1
17.4	18.2	4.6
17.1	19.8	15.8
18.4	23.1	25.5
17.6	19.9	13.1
Average.......		14.8
l-Tyrosine, 1.5 gm.		
13.1	25.5	94.6
13.3	15.7	18.1
16.3	18.0	10.4
14.1	21.2	50.3
15.6	13.1	
16.3	15.4	
16.7	20.8	24.5
15.2	22.4	47.3
Average.......		30.7

Column group 3

2 day control period	2 day experimental period	Increase
d-Glutamic acid, 1.5 gm.		
mg.	mg.	per cent
14.7	18.6	26.5
17.7	15.0	
11.8	16.9	43.2
15.8	23.5	48.7
12.4	23.0	85.4
17.7	18.8	6.2
16.8	16.5	
16.2	16.5	
14.1	16.3	15.6
Average.......		25.1
Histidine, 1.0 gm.		
15.0	17.5	16.6
15.4	13.5	
16.7	18.1	8.5
15.6	18.8	20.5
18.0	23.2	28.9
17.7	25.2	42.3
Average.......		19.5
l-Cystine, 1.5 gm.		
11.5	16.1	40.0
11.8	17.5	48.3
14.7	19.6	33.3
16.7	18.5	10.8
18.4	22.0	19.5
18.6	20.0	7.5
16.6	21.6	30.1
17.0	23.0	35.3
Average.......		28.1

of dilute HCl was used to collect the 24 hour specimen of urine on the plate. The whole was taken up with more water and then transferred to a 100 cc. graduate. This process was repeated twice more, and the washings added to the original. Finally the whole was made up to 50 cc., thoroughly shaken, and filtered. A 5 cc. sample of the filtrate was used for the determination of creatinine by the method of Folin (16).

The creatinine excretion was determined daily. Results for 2 days were then added together to obtain a 2 day output. On the morning of the 3rd day 1 or 1.5 gm. of a purified amino acid were either fed as such or mixed with a small amount of the stock diet. The supplement was usually consumed in a few hours, during which time the stock diet was removed from the cage. The creatinine was determined as described above.[4]

The results obtained in this study are given in Table V. It is again observed that the feeding of all the amino acids studied caused increases in the creatinine output somewhat similar to those obtained in the muscle studies reported above. The average per cent increases, from lowest to highest, in creatinine excretion were aspartic acid 14.2, valine 14.8, histidine 19.5, glutamic acid 25.0, arginine monohydrochloride 26.8, cystine 28.1, tyrosine 30.7, and glycine 35.9.

Creatinine Elimination in Urine of Man

It is a debatable question whether the results of animal experimentation can be applied directly to man. In order to obtain the facts we next secured the cooperation of several graduate students, laboratory assistants, and members of the staff, including ourselves, to serve as experimental subjects.

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[4] The amino acids, if excreted as such in these studies, were not the cause of the increased creatinine values obtained. This was shown in several experiments as follows: 10 cc. of the urine filtrate were divided into two equal parts. In one part the creatinine was determined as usual, and in the other 0.2 gm. of the amino acid in question was dissolved, and the creatinine determined. There was an increased reading on the colorimeter in every case, which indicated that the amino acids interfered with the color development, and lower results for creatinine would have been obtained in these cases. Since practically every experimental urine gave values higher than normal for creatinine, it is evident that these values were due to metabolized and not to excreted amino acids.

All subjects were in good health and were instructed to live on a meat-free diet during the period of study. The daily creatinine output was determined for several days before and after ingestion

TABLE VI

Effect of Feeding Proteins and Amino Acids upon Creatinine Elimination in Man

Subject	Average control creatinine excretion	Experimental creatinine excretion	Increase	Meat-free diet plus
	gm.	*gm.*	*per cent*	
H.H.B.	1.63	2.00	22.0	100 gm. casein
	1.63	2.60	58.5	100 " "
	1.63	2.00	22.0	100 " edestin
	1.63	2.00	22.0	100 " fruit Jello
	1.63	1.94	12.2	(2nd day)
	1.63	2.07	26.2	8 gm. glycine
	1.63	2.40	46.3	(2nd day)
	1.63	1.98	44.5	(3rd ")
	1.63	2.07	26.8	(4th ")
	1.63	2.56	56.1	8 gm. glycine
	1.63	1.44	0.0	8 " "
	1.63	2.24	37.2	10 " alanine
	1.63	1.98	20.7	(2nd day)
	1.63	2.25	37.3	(3rd ")
	1.63	1.95	12.8	(4th ")
	1.63	2.01	22.5	5 gm. arginine HCl
	1.63	2.33	42.1	(2nd day)
	1.63	2.21	34.7	5 gm. arginine HCl
	1.63	1.91	16.4	(2nd day)
G.D.M.	1.17	1.90	62.4	100 gm. casein
	1.17	1.31	11.9	100 " edestin
	1.17	1.39	18.7	100 " fruit Jello
G.G.	1.33	1.95	47.3	8 gm. glycine
	1.33	1.80	·35.3	8 " "
R.F.H.	1.77	1.96	10.7	10 gm. glycine
	1.77	2.05	15.8	10 " "

of the supplement. One of the following substances, dissolved or dispersed in milk, was taken for lunch on the experimental day: 100 gm. of casein, 100 gm. of edestin, 100 gm. of fruit Jello,

TABLE VII

Determination of Excretion of Creatinine in Normal Urine and after Ingestion of 100 Gm. of Casein with Milk*

Subject	Normal creatinine excretion	Average	Experimental creatinine excretion	Average	Increase
	gm.	*gm.*	*gm.*	*gm.*	*per cent*
W.B.D.	1.16		1.62		
	1.19	1.18	1.59	1.61	36
R.W.S.	1.89		2.41		
	1.83		2.55	2.48	33
	1.88	1.87			
L.K.B.	1.60				.
.	1.72		1.51		
	1.33	1.55	1.21	1.36	None
D.A.C.	1.67				
	1.65		1.62		
	1.52	1.61	1.44	1.53	None
H.L.K.	1.25				
	1.19		1.59		
	0.99	1.14	1.67	1.63	43
S.W.	1.38				
	1.32		1.62		
	1.13	1.28	1.52	1.57	23
I.B.T.	1.13				
	1.04		2.16		
	1.16	1.11	1.22	1.69	52
W.W.B.	1.48				
	1.30		1.89		
	1.33	1.37	1.78	1.84	34
M.F.C.	1.41				
	1.58		1.62		
	1.42	1.47	2.36	1.99	35

* Experiment performed by W. Deichmann.

8 gm. of glycine, 10 gm. of alanine, or 5 gm. of arginine mono-hydrochloride.

The results of this study are given in Table VI. In the case of one of us (H.H.B.) upon whom most of the experiments were carried out, it is clearly seen that the ingestion of proteins and amino acids gave good increases above the average control output (1.63 gm. of creatinine daily). For instance following 8 gm. of glycine the increases were 0.0, 46, and 56 per cent. Thus in two out of three experiments results were obtained with this amino acid similar to those obtained by Brand *et al.* (6) in cases of muscular dystrophy, but it can be said here that no subject of this study was suffering from any known pathological condition. It would seem that the normal human adult would serve as a good experimental subject in experiments of this kind, *but only if large amounts of supplements are taken at one time.* Somewhat similar results were obtained with the other subjects.

Table VII contains the results obtained by Mr. Wilhelm Deichmann working independently in this laboratory, with nine dental students as subjects. It is seen that in seven out of nine there were large increases in creatinine excretion after the ingestion of 100 gm. of casein dispersed in milk.

<div align="center">DISCUSSION</div>

Creatine Formation from Amino Acids of the Diet—The results obtained in these studies, together with the evidence in the recent literature to be cited below, leave no doubt but that creatine may arise as a result of feeding proteins and amino acids. It is also true that many negative experiments have been reported in regard to these substances. It is well known that on a moderately high protein diet there is little if any increased formation of creatine. This being the case, the chief point the writer wishes to emphasize is that the intensity of the protein or amino acid metabolism, per unit of time, must be speeded up to a large extent, exceeding normal metabolic reactions if creatine formation in excess of normal is to be obtained. The feeding of proteins or amino acids as such in large quantities for 1 day usually gives the largest increases in creatine formation and in creatinine excretion. It may well be that if other constituents of the diet, such as fat, carbohydrate, salts, etc., be present, then no increased creatine formation results. In other words it is necessary to accelerate the protein or amino acid metabolism considerably in order to obtain the increases.

Objection might be raised to feeding such large amounts of amino acids in these studies. But these amounts were only fed once and no kidney damage resulted, and we do not believe that the above procedure can be considered as unphysiological.

The results of this study do not tell us the mechanism of creatine formation on a normal protein diet, but they do indicate that creatine must either be formed from the amino acids if they are fed in large amounts, or that the amino acids must stimulate creatine-creatinine metabolism in some way other than by their specific dynamic action. We are inclined to favor the former view.

The theory of creatine formation from amino acids is by no means new. Harding and Young (2) suggested that creatine may be derived from cystine. Gross and Steenbock (3) confirmed the effect of arginine and cystine. Zwarenstein (17), with his subject A, found an increase of 9.2 per cent in creatinine elimination after giving 10 gm. of glycine and 12.8 per cent after giving 20 gm. of alanine, yet he attached no importance to these results. Bergmann and Zervas (4) synthesized glycocyamine and creatine from arginine derivatives and glycine, and they believe that creatine arises in the body from the precursors of glycine and arginine. Brand and coworkers (6) have demonstrated, in patients suffering from muscular dystrophies, that glycine caused a 40 per cent increase in creatinine elimination. Later (18) they reported that gelatin and, to a lesser extent, edestin feeding had much the same effect. In our experiments reported in this paper glycine caused the largest increases in creatinine elimination in rat urine of any amino acid studied. In the case of one of us (H.H.B.) as subject, an average increase of 51 per cent in creatinine elimination was obtained after ingesting this amino acid.

Steudel and Freise (19) injected 8.6 gm. of histidine into a dog, causing an increased excretion of creatinine of 86.3 and 68.9 per cent the day after injection, but they concluded that creatinine did not originate from histidine. Schumann (20) obtained no increase after giving histidine. Abderhalden and Buadze (7) criticized the work of Schumann, due to the fact that his doses were too small to be expected to give increases in creatinine excretion. Abderhalden and Buadze found increases after giving both nucleic acid and histidine.

Arginine, of all the amino acids, has received the most attention

as a mother substance of creatine since it is related chemically to this substance through its guanidine grouping. The theory of Czernecki (21), later adopted by Knoop (22) and Neubauer (23), would picture arginine as being transformed into guanidinebutyric acid, then into guanidineacetic acid, which upon methylation would yield creatine. With the exception of the second substance definite evidence is given in this paper to show that both arginine and glycocyamine may be transformed into muscle creatine. The work of Thompson (24) with regard to arginine is very convincing to us. Scull and Rose (25) have shown that arginine can be synthesized in the body, and it is possible that this endogenous arginine may be the mother substance of endogenous creatine.

Koch (26) was the first to suggest that creatine, as a methylated amino acid, might be related to choline, a methylated amino alcohol. This view was confirmed by Riesser (27), Shanks (28), and Abderhalden and Möller (29). Choline hydrochloride caused the usual increases in creatine formation in the present studies but negative results were obtained with sarcosine, betaine hydrochloride, and nucleic acid.

Creatine formation from amino acids also receives support from the data of comparative biochemistry. The complex basic extractives, such as creatine of vertebrate muscle, are largely replaced by simple amino acids in invertebrate muscle. Thus Chittenden (30) was the first to find glycine in scallop muscle and Wilson (31) showed that 53 per cent of the nitrogen of this type of muscle was monoamino nitrogen. Ackermann and Kutscher (32) and Suzuki and coworkers (33) isolated leucine, tyrosine, and alanine, while taurine was isolated by Mendel (34), Mendel and Bradley (35), and Henze (36) from related forms. Meyerhof (37) has recently shown that the muscles of several genera of invertebrates contain an "arginine-phosphagen" in place of phosphocreatine of vertebrate muscle.

Relationship between Intensity of Protein Metabolism and Creatine-Creatinine Metabolism—It is well known that diets rich in protein may produce creatinuria in infants and children. Daniels and Hejinian (38) concluded that the creatinuria of infancy is related to the ingestion of protein and that an increase in urinary nitrogen is coexistant with an increase in urinary creatine. Somewhat the same conclusions were reached by Wang and coworkers

(39, 40). Terroine and Danmanville (41) stated that the classi-fication of proteins according to their creatinogenic value is exactly inverse to that of their biological value. Recent evidence by Bollman (42) would also tend to show that the level of protein metabolism is of great influence upon the creatine-creatinine transformation in the body. Our results confirm this view.

Effect of Specific Dynamic Action of Proteins and Amino Acids upon Creatine-Creatinine Metabolism—We do not believe that creatine formation in the present studies was the result of the specific dynamic action of the amino acids. Rapport and Beard (43) have shown that histidine and valine exerted no increase in heat production in the dog, while these two amino acids caused large increases in muscle creatine. A comparison of the specific dynamic action of the amino acids per gm. ingested with the average per cent increase in creatine per gm. of muscle tissue, glycine being taken as 100 in both cases, is given in Table IV. It is seen that creatine production from the amino acids was much greater than their specific dynamic action. Tissue protein exerts no specific dynamic effect and there is good reason to believe that tissue arginine or glycine may each take part in creatine formation. Proteins also differ in their ability to form creatine, while showing similar specific dynamic effects. Furthermore the specific dy-namic action of an amino acid manifests itself within a few hours after ingestion, while increased creatine formation and creatinine elimination are taking place as long as 2 or 3 days after amino acid feeding.

Endogenous Origin of Creatine and Effect of Muscle Mass and Body Weight upon Creatinine Metabolism—It is possible that the results obtained in the present studies may be explained on the assumption that there was a general stimulation of the endogenous metabolism with a resultant formation of creatine. The following evidence, however, will hardly support this view.[5]

Garot (44) concluded that creatinine was of endogenous origin and that creatine was of both exogenous and endogenous origin. Later (45) he concluded that creatine was of exogenous origin. Terroine and his associates (46) later concluded that the creatinine excretion was not to be considered as a true index of the endoge-

[5] Inthis connection see Mitchell and Hamilton (9) p. 491.

nous metabolism.   In their most recent study of this question, Terroine, Bonnet, Danmanville, and Mourot (47) gave the following conclusions: (1) The excretion of creatinine remains constant though that of the endogenous nitrogen varies greatly; (2) the excretion of creatine follows that of the total nitrogen; (3) creatine rises with a rise of excretion of the endogenous nitrogen as influenced by the diet; (4) the creatinic bodies behave in endogenous nitrogen metabolism exactly as in exogenous nitrogen metabolism.

Garot (44) also stated that creatinine excretion was not proportional to the muscular mass.   Terroine and Garot (48) studied the creatinine excretion in various warm blooded animals with heat productions of very different intensities.   The daily creatinine output of the rat was 60 mg. per kilo as compared to 16 mg. per kilo for the horse.   It was also observed that the creatinine excretion per kilo presents very great differences among warm blooded animals.   Evidence was also obtained by one of us (H.H.B.) (49) showing the lack of relation between creatinine excretion and several body measurements.

Creatinuria is usually present in phlorhizin diabetes, and it is generally believed that there is a close relationship between creatine metabolism and carbohydrate metabolism.   Benedict and Osterberg (50) fed a creatine-free protein diet to completely phlorhizinized dogs and studied its effect upon creatine elimination.   They concluded that a high creatine elimination may be maintained which is wholly independent of the body tissue destroyed.   The creatine eliminated did not have its origin in the preformed creatine of muscular tissue.   In this connection, Paton (51) stated that the ingested protein could furnish an abundant source of the guanidine required for creatine formation.   These conclusions are not inconsistent with our view that creatine may arise from the exogenous protein of the diet.

<div style="text-align:center">SUMMARY AND CONCLUSIONS</div>

Proteins, amino acids, and related substances have been fed as such to young rats and their influence upon creatine formation in the muscles studied.   Amino acids have also been fed to adult rats and their effect upon creatinine elimination determined.   Finally, proteins and amino acids were fed to several students and members of the laboratory staff and the influence of these sub-

stances upon creatinine excretion observed. The results obtained were as follows:

1. The average creatine content of the muscles of young rats of about 50 gm. of body weight was 4 mg. per gm.

2. The feeding of d-arginine monohydrochloride, histidine, d-glutamic and l-aspartic acids, l-cystine, l-tyrosine, dl-phenylalanine, dl-alanine, dl-valine, glycocyamine, choline hydrochloride, creatine, casein, and edestin, each, has the ability greatly to increase the creatine content of young rat muscle. The average per cent increases above normal ranged from 12.5 for alanine to 37.5 for cystine.

3. All of the amino acids studied brought about an increased daily elimination of creatinine in the urine of adult rats. The average per cent increases above normal ranged from 14.2 for aspartic acid to 36 for glycine.

4. Proteins or amino acids, fed in comparatively large amounts at one time, greatly increased the daily creatinine output in normal human subjects.

5. It is only those amino acids of the protein molecule that are able to give rise to creatine, since synthetic amino fatty acids had no effect in this connection.

6. Creatine formation and creatinine excretion were either due to the feeding of large quantities of proteins and amino acids at one time, or these substances stimulated creatine-creatinine metabolism, in some way other than by specific dynamic action, with a resulting increased formation of creatine in the muscles and elimination of creatinine in the urine.

BIBLIOGRAPHY

1. Hunter, A., Creatine and creatinine, London and New York (1928).
2. Harding, V. J., and Young, E. G., J. Biol. Chem., 41, p. xxxvi (1920).
3. Gross, E. G., and Steenbock, H., J. Biol. Chem., 47, 33 (1921).
4. Bergmann, M., and Zervas, L., Z. physiol. Chem., 172, 277 (1927).
5. Bergmann, M., and Köster, H., Z. physiol. Chem., 159, 179 (1926).

6. Brand, E., Harris, M. M., Sandberg, M., and Ringer, A. I., *Am. J. Physiol.*, **90**, 296 (1930).
7. Abderhalden, E., and Buadze, S., *Z. physiol. Chem.*, **189**, 65 (1930).
8. Thomas, K., *J. Nutr.*, **2**, 419 (1930).
9. Mitchell, H. H., and Hamilton, T. S., The biochemistry of the amino acids, American Chemical Society Monograph No. 48, New York, 342 (1929).
10. Rose, W. C., Helmer, O. M., and Chanutin, A., *J. Biol. Chem.*, **75**, 543 (1927).
11. Cox, G. J., *J. Biol. Chem.*, **78**, 475 (1928).
12. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **78**, 627 (1928).
13. Chanutin, A., and Silvette, H., *J. Biol. Chem.*, **80**, 589 (1928).
14. Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, **25**, 195 (1916).
15. Jackson, C. M., and Lowrey, L. G., *Anat. Rec.*, **6**, 449 (1912), cited by Donaldson, H. H., The rat, Philadelphia, 2nd edition, 185 (1924).
16. Folin, O., *J. Biol. Chem.*, **17**, 469 (1914).
17. Zwarenstein, H., *Biochem. J.*, **22**, 307 (1928).
18. Brand, E., Harris, M. M., Sandberg, M., and Lasker, M. M., *J. Biol. Chem.*, **87**, p. ix (1930).
19. Steudel, H., and Freise, R., *Z. physiol. Chem.*, **120**, 244 (1922).
20. Schumann, R., *Z. physiol. Chem.*, **186**, 104 (1930).
21. Czernecki, W., *Z. physiol. Chem.*, **44**, 294 (1905).
22. Knoop, F., *Z. physiol. Chem.*, **67**, 489 (1910).
23. Neubauer, O., in Abderhalden, E., Biochemisches Handlexikon, Berlin, **4**, 360 (1911).
24. Thompson, W. H., *J. Physiol.*, **51**, 111, 347 (1917).
25. Scull, C. W., and Rose, W. C., *J. Biol. Chem.*, **89**, 109 (1930).
26. Koch, W., *Am. J. Physiol.*, **15**, 15 (1905).
27. Riesser, O., *Z. physiol. Chem.*, **86**, 415 (1913).
28. Shanks, W. F., *J. Physiol.*, **55**, p. viii (1921).
29. Abderhalden, E., and Möller, P., *Z. physiol. Chem.*, **170**, 212 (1927).
30. Chittenden, R. H., *Ann. Chem.*, **178**, 266 (1875).
31. Wilson, D. W., *J. Biol. Chem.*, **17**, 385 (1914).
32. Ackermann, D., and Kutscher, F., *Z. Untersuch. Nahrungs- u. Genussmittel*, **13**, 180 (1907), cited by Wilson (31).
33. Suzuki and coworkers, *J. Coll. Agric., Imp. Univ. Tokyo*, **5**, 1 (1912), cited by Wilson (31).
34. Mendel, L. B., *Beitr. chem. Physiol. u. Path.*, **5**, 582 (1904), cited by Wilson (31).
35. Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, **17**, 167 (1906).
36. Henze, M., *Z. physiol. Chem.*, **43**, 477 (1905).
37. Meyerhof, O., *Arch. sc. biol.*, **12**, 536 (1928).
38. Daniels, A. L., and Hejinian, L. M., *Am. J. Dis. Child.*, **38**, 499 (1929).
39. Wang, C. C., and Kaucher, M., *Am. J. Dis. Child.*, **38**, 468 (1929).
40. Wang, C. C., Hawks, J. E., Huddleston, B., Wood, A. A., and Smith, E. A., *J. Nutr.*, **3**, 79 (1929).
41. Terroine, E. F., and Danmanville, P., *Compt. rend. Acad.*, **188**, 1439 (1929).

42. Bollman, J. L., *J. Biol. Chem.*, **85**, 169 (1929–30).
43. Rapport, D., and Beard, H. H., *J. Biol. Chem.*, **80**, 413 (1928).
44. Garot, L., *Rev. franç. pédiat.*, **6**, 265 (1930).
45. Garot, L., *Arch. internat. physiol.*, **29**, 65 (1927).
46. Terroine, E. F., Bonnet, R., Chotin, R., and Mourot, G., *Arch. internat. physiol.*, **33**, 60 (1930).
47. Terroine, E. F., Bonnet, R., Danmanville, P., and Mourot, G., *Compt. rend. Acad.*, **191**, 1473 (1930).
48. Terroine, E. F., and Garot, L., *Arch. internat. physiol.*, **27**, 69 (1926).
49. Beard, H. H., unpublished data.
50. Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, **18**, 195 (1914).
51. Paton, N., *Rep. Brit. Assn. Adv. Sc.*, 294 (1919), cited by Hunter (1) p. 210.

STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

I. INFLUENCE OF IRON UPON BLOOD REGENERATION*

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Iron therapy in the treatment of certain forms of anemia has witnessed a succession of opposing theories as to the most suitable form of iron administration: organic *versus* inorganic iron, soluble *versus* insoluble iron, ferric *versus* ferrous iron, and now iron alone *versus* iron with other inorganic supplements.   The literature on the subject is very large and will not be discussed here, since the excellent reviews of Hall (1), Meyer (2), and Robscheit-Robbins (3) are available.

Introduction during the past few years of several methods of producing experimental anemia has given considerable impetus to this line of investigation.   Some of the most important recent contributions are those of Whipple, Robscheit-Robbins, and associates (4–6), Hart, Steenbock, and associates (7–11), and Mitchell and coworkers (12–14).   Mitchell's work clearly showed that the deficiency in milk which was responsible for the anemia was inorganic in nature, rather than organic, since the anemia could be cured by the addition of inorganic iron salts.   In the publications

of the Wisconsin investigators (9, 11) very definite evidence appears to have been given that inorganic Fe salts, *e.g.* the chloride, acetate, sulfate, etc., prepared from pure reagents, will not correct the nutritional anemia of their animals, while such foods as cabbage and lettuce, the ash and alcoholic extract of the same, the ashed residues from dried beef liver, yellow corn, and their extracts, with Fe, will cure the condition.   In their first paper (7), they stated that some organic factor was necessary in addition to Fe.   Later, however, since excellent recoveries were also obtained from the ash of different plant extracts (8), they came to the conclusion that the active factor with Fe in hemoglobin regeneration was inorganic in character rather than organic.

Although the animals of Hart, Steenbock, and their coworkers were anemic as a result of the Fe deficiency in the milk, practically no effect upon blood regeneration was observed when pure Fe salts were given.   They (8, 11) suggest that the beneficial results obtained by Mitchell and Schmidt (12) and ourselves (15, 16), in anemia studies, may have been due to impurities, such as Cu, in the Fe salts used.

In view of the fact that there still exists a difference of opinion as regards the beneficial effect of Fe in the nutritional anemia of the rat, and in order to establish basal conditions for the testing of other elements in this connection, Fe in several different doses has been fed daily to rats rendered anemic by milk feeding. The results of these experiments are given below.

Experimental Methods

Young rats, 23 to 25 days of age, were rendered anemic by feeding whole milk for a period of 5 to 8 weeks.   It was found that the length of time necessary to bring the animals to a marked anemic level depended upon the length of time they had had access to their mother's diet.   The stock animals, from which a very large number of the experimental animals were secured, were fed upon Sherman's Diet B ($\frac{2}{3}$ whole wheat flour and $\frac{1}{3}$ whole milk powder) together with $CaCO_3$ and NaCl, each to the extent of 1 per cent of the weight of the wheat, with fresh lettuce three times a week.   Many young animals were also secured from stock rats kept on Maynard's calf meal diet with lettuce, and from rats whose diet was unknown to us.   This was done in part to secure rats

raised under a variety of conditions. Most of the animals, after recovery from the anemia, were placed on Sherman's Diet B and were the parents of future experimental rats. No difficulty was experienced in bringing the animals to a marked anemic level in about 6 weeks of milk feeding.

When the erythrocyte count was about 2 to 3 million per c.mm., and the hemoglobin content about 2 to 4 gm. per 100 cc., additions of $FeCl_3$ (0.08 to 2.5 mg. of Fe) were fed daily in the milk.

Iron—Baker's Fe wire for standardizing, stated to be 99.8 per cent pure, was used in the experiments with the first 203 animals. The J. T. Baker Company gave the Cu content of this particular sample, determined by special analysis, to be 0.0018 per cent. This being the case, the 0.5 mg. of Fe fed daily would contain 0.001 mg. of total impurities, and approximately 0.0001 mg. of Cu.

In experiments with Rats 204 to 256, a sample of iron wire obtained from the Bureau of Standards (thought to contain about 0.01 per cent of both Ni and Cu) was used. In the 0.5 mg. of Fe fed daily, 0.00005 mg. of both Cu and Ni would be introduced, an amount obviously too small to exert any effect on hemoglobin regeneration.

In experiments with Rats 257 to 497, a sample of electrolytic iron (probably the purest form of iron obtainable) from the J. T. Baker Company, was employed, the filtrate being used after treatment with H_2S. Rats 498 to 552 received electrolytic Fe ($FeCl_3$) supplied by Dr. W. P. Davey of Pennsylvania State College. Groups of anemic rats were also run on Fe solutions furnished by Drs. C. A. Elvehjem and W. E. Krauss.

Preparation of Fe Solutions—The $FeCl_3$ solutions were prepared from the above samples of Fe by dissolving 2 gm. of the wire or metal in concentrated HCl, with the aid of heat and a very small amount of concentrated HNO_3, evaporating to dryness, the HNO_3 being removed by repeating the solution and evaporating twice more with HCl. The final residue was dissolved in the least amount of HCl, and then diluted to the mark in a volumetric flask with water redistilled from glass to contain 2 mg. of Fe per cc. In some other experiments the solutions were prepared exactly as described by Hart and coworkers (9) going through the stage of $Fe(OH)_3$, etc.

Caging—The young rats were kept in galvanized iron cages with false bottoms (Army Medical School model).   It should probably be noted that these cages were covered with aluminum paint and aluminum has no effect with Fe on blood regeneration. To rule out the possibility of any supplementary elements being taken up from these cages, a series of rats was kept on the milk-Fe diets in glass funnel cages, or in large glass museum jars.   The milk was fed in porcelain ointment jars.   In all cases identical results with the original Fe experiments were obtained.

Source of Rats—In addition to the two sources of rats mentioned above, we also secured young animals from the colony of the Institute of Pathology, and from two breeders in this city and one in Philadelphia.   All of the 750 young rats used in this series of studies, with eight exceptions, developed anemia, and practically all of them recovered upon different supplements.   We have noted, however, that our strain of pied rats gave much more regular responses in hemoglobin regeneration and increase in body weight than the albinos.

Milk Used—Grade A milk from three dealers of this city was used.   Most of the milk came from a distance of at least 50 miles east of Cleveland and this would seem to remove the source of the milk from the industrial atmosphere of the western part of the city.   It was usually obtained from the cows by mechanical milkers, caught in monel metal containers, and immediately taken to the cooler.   It was then poured through about 5 feet of copper pipe into a large nickel cooler, kept at 7° for 24 hours, bottled, and delivered to the laboratory.   Analysis of this milk by the method of Elvehjem and Lindow (17) showed 0.44 mg. of Cu per liter.   It would appear, therefore, that the milk used in these experiments did not contain more than the usual amount of Cu.

In the experiments with Rats 196 to 256, the milk never came in contact with any metal containers.   The cows were milked into earthenware jars, the milk bottled, and at once brought to the laboratory.   The same results were obtained as with the milk carried through the customary processes.

Blood Counts and Hemoglobin Estimations—At the end of 6 weeks of milk feeding, a few drops of blood, obtained by clipping the end of the tail, were used for the red blood cell count and the determination of hemoglobin.   After taking the sample and after the bleed-

ing had stopped, the end of the tail was dipped in alcohol. In no case was gangrene noticed. If the blood could not be obtained from the end of the tail, it was secured by puncturing the caudal vein. In the winter the tail was dipped in warm water before the blood was taken, but this treatment was unnecessary at other times of the year. Red blood cell counts were made weekly with a Levy counting chamber with double Neubauer ruling which was checked with an American standard hemocytometer certified by the Bureau of Standards. Hemoglobin estimations were also made weekly by the acid hematin method, color comparison being made with a calibrated Newcomer disc in a Klett biocolorimeter.

In many cases the hemoglobin content was so low after 6 weeks of milk feeding that color comparison was impossible. In such instances estimated values were used for all figures below 3 gm. of hemoglobin per 100 cc. The advantage of including red blood cell counts in nutritional anemia studies is thus evident, since with a large number of our animals, the severity of the condition at the beginning of the experiments could not be ascertained with the acid hematin method. In some of the later experiments the very sensitive colorimetric benzidine method of Bing and Baker (18) for hemoglobin was employed. Values of 7 to 8 million erythrocytes per c.mm. and 13 to 14 gm. of hemoglobin per 100 cc. are considered normal in these studies.

Results

145 anemic young rats have been fed daily on doses of Fe from 0.08 to 2.5 mg. Representative results obtained with 73 animals are given in Table I.

When 0.08 and 0.16 mg. of Fe were given daily there was some response in hemoglobin regeneration, while in six out of eight experiments the erythrocyte recovery took place in an average time of about 6 weeks. These doses must, therefore, be too small for hemoglobin regeneration on Fe alone in the standard time of 6 weeks.

A different result was obtained when 0.25, 0.32, or 0.40 mg. of Fe was fed daily. On all these doses blood regeneration was complete in about 6 weeks for hemoglobin and 4.6 weeks for red blood cells. There were two or three animals on the 0.25 mg.

Influence of Whole Milk Plus 0.08 to 2.0 Mg. of Iron Daily upon the Nutritional Anemia of the Rat

Amount of Fe added daily	Rat No.	Body weight		Hb per 100 c̊c.		R.b.c. per c.mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b.c.
mg.		*gm.*	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*
0.08	350	68	97	2.0	6.5	2.2	7.3	10	7
	351	77	106	2.5	5.0	2.5	7.3	10	7
	408	82	116	2.8	7.7	3.9	7.7	8	5
	409	101	129	2.9	7.7	2.6	7.8	8	5
0.16	352	48	95	3.0	6.0	2.5	7.3	8	7
	353	98	139	3.0	12.5	3.6	8.1	7	4
	410	75	136	2.6	7.7	2.4	3.7	6	6
	411	82	120	2.6	8.7	3.0	5.1	6	6
0.25	354	70	102	3.0	13.6	3.0	7.5	6	5
	355	64	133	3.0	13.4	2.5	8.1	6	5
	412	77	109	2.9	14.2	2.9	7.6	6	4
	413	71	111	2.6	9.5	2.8	8.4	6	4
	452	96	120	2.5	13.1	1.7	8.2	6	5
	453	77	106	3.0	11.7	2.1	8.2	6	6
	454	86	102	3.0	13.6	2.0	7.1	6	4
	455	84	107	2.0	7.7	3.0	8.6	6	4
	456	109	122	3.1	13.6	3.6	8.3	6	4
Average.........		81.5	112.4	2.8	12.2	2.5	8.0	6.0	4.6
0.5	53	87	105	6.7	14.9	3.9	8.8	5	4
	54	105	125	6.4	14.0	5.7	7.9	6	4
	55	120	163	5.7	14.6	5.5	7.9	7	3
	56	86	149	4.6	13.7	3.4	8.2	6	4
	57	115	168	5.3	14.1	4.7	8.1	6	5
	58	92	119	4.3	13.6	4.3	7.9	6	6
	65	87	145	4.0	13.3	4.2	8.7	6	4
	66	115	140	4.0	13.3	2.0	9.6	6	4
	67	87	120	3.9	14.0	4.0	9.2	7	4
	97	62	128	5.0	14.2	2.4	8.3	6	3
	98	84	139	4.6	14.0	2.4	9.1	6	3
	99	69	140	4.2	14.6	1.8	8.1	6	4
	100	68	115	3.6	13.8	3.5	7.8	6	3
	107	73	115	4.1	13.1	2.8	8.1	6	3
	116	73	126	3.1	13.6	4.0	8.2	6	4
	147	66	111	6.3	14.1	3.1	7.8	6	3
	148	57	97	6.2	13.6	4.6	8.9	6	3
	149	61	107	5.0	14.2	3.9	8.4	6	4
	150	65	113	4.6	14.0	1.7	8.4	6	4
Average.........		82.7	127.6	4.8	13 9	3.6	8.4	6	3.8

Amount of Fe added daily	Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b.c.
mg.		*gm.*	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*
0.5 (H₂S fil-trate)	98	57	97	6.2	13.6	4.6	8.9	6	3
	99	61	107	5.0	14.2	3.9	8.4	6	4
	100	61	113	4.6	14.0	2.8	8.4	5	3
	264	76	100	5.1	13.3	1.9	7.6	5	4
	307	75	108	4.8	13.2	2.4	8.1	6	4
	308	71	116	2.5	13.3	2.1	9.0	5	4
	340	101	139	3.6	13.3	3.6	7.9	6	3
	341	104	151	2.2	12.5	2.2	7.6	6	4
	342	107	146	3.0	12.5	3.1	8.0	6	4
	343	117	162	3.0	13.3	3.1	7.7	6	4
	344	103	124	3.3	16.1	3.4	8.0	6	4
	345	93	118	2.0	14.3	2.1	7.9	6	4
Average..........		85.5	123.4	3.8	13.6	2.9	8.1	5.7	3.7
1	47	89	110	5.7	14.9	4.2	8.5	5	4
	48	98	136	5.1	14.3	4.9	8.9	5	2
	49	95	168	5.0	13.5	3.8	7.8	6	5
	50	131	167	5.2	14.4	3.9	8.3	5	3
	51	123	165	5.1	14.4	4.8	8.2	5	4
	52	83	108	6.5	13.6	5.6	8.1	4	3
	62	116	149	7.4	13.6	5.3	8.3	4	3
Average		105	143	5.7	14.1	4.6	8.3	4.9	3.4
1.5	68	79	110	3.8	14.3	3.1	8.0	2	3
	69	104	118	4.2	13.8	4.1	9.0	3	4
	70	102	135	4.2	14.1	2.9	8.9	4	3
Average..........		95	121	4.1	14.1	3.4	8.6	3	3.3
2.0	168	66	98	6.6	16.6	3.7	9.2	2	1
	169	58	79	5.5	15.3	2.8	10.5	2	2
	170	62	74	4.1	12.8	3.7	8.2	1	1
	171	65	97	3.7	14.3	2.3	9.1	2	2
	172	71	96	3.0	16.4	2.1	8.1	2	2
	173	66	90	3.0	16.6	2.0	10.8	2	2
	181	60	79	3.5	16.1	2.3	9.7	2	2
	182	66	93	3.5	13.3	3.1	8.1	2	2
	183	68	94	3.0	17.3	3.0	8.7	2	2
	184	58	72	3.7	14.3	4.3	8.0	1	1
	185	61	88	3.0	14.1	3.6	9.8	2	2
	186	55	78	3.8	15.2	3.4	10.6	2	2
Average		63	86.5	3.9	15.2	3.0	9.2	1.8	1.8

dose, however, which did not recover the normal amount of hemoglobin in this time, but since the great majority showed recoveries, it would appear that these were exceptional cases. Although two rats died on the 0.32 mg. dose, none died on the 0.08 and 0.16 mg. doses, and one might conclude that these deaths were due to the severe anemic condition of the animals at the beginning of the experiment, rather than to a lack of response to the Fe.   The four rats which received 0.08 mg. of Fe daily were still alive after 14 weeks, showing that this small amount of Fe is capable of prolonging the life of the animal, but is insufficient for blood regeneration.

95 anemic young rats fed daily on 0.5 mg. of Fe (in the form of the chloride, prepared from standard iron wire, electrolytic Fe, and the H_2S filtrates from the same) showed average recovery of hemoglobin in 6 weeks, and of cells in less than 4 weeks.   These rats were used as litter mate controls for the experimental animals given in Paper II of this series.   No trouble has been experienced in repeating these observations at different intervals during the past 3 years.

The time of recovery was found to be directly proportional to the amount of Fe fed daily, up to 2 mg.   On this dose practically all the animals attained a value of 10 or 11 gm. of hemoglobin the 1st week, and 4 to 6 million erythrocytes per c.mm. in the same length of time.   At the end of the 2nd week several counts of 9 to 11 million per c. mm. (up to 2 million above normal) were observed. There was no quicker response when 2.5 mg. of Fe (H_2S filtrate of electrolytic Fe) were fed daily.   Thus, 2 mg. daily would appear to be the smallest amount of Fe which will give the maximal blood regeneration.

During the course of these studies, Dr. F. C. Bing of our staff was asked to conduct, independently, experiments on a group of animals given Fe alone.   The experiments were run in specially constructed glass cages (large neck, 12 liter bottles, with a cross mesh bottom made by fusing glass tubing).   The results he obtained are given in Table II.   It will be noted that hemoglobin and erythrocyte recovery took place in the usual time.

In the fall of 1930 we were kindly supplied with Fe solutions by Drs. C. A. Elvehjem and W. E. Krauss, of the Wisconsin Laboratory and Ohio Agricultural Experiment Station, respectively.

TABLE II

*Influence of Inorganic Iron upon Blood Regeneration in Nutritional Anemia**
Experiment of Dr. F. C. Bing.

Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery	
	Before	After	Before	After	Before	After	Hb	R.b.c.
	gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*
A 1	110	151	3.6	13.6	4.1	8.9	6	4
A 2	102	156	4.0	13.6	4.9	10.4	6	4
A 3	99	157	3.5	13.4	3.6	9 6	6	4
A 4	92	157	5.0	12.7	4.1	10.7	6	4
A 5	102	153	6.2	13.0	4.9	10.5	6	4
A 6	98	149	3.8	12.9	4.3	10.2	6	4
Average..	100.5	154.0	4.4	13.2	4.3	10.1	6	4

* Rats A 1, A 2, and A 3 received 0.5 mg. of Fe daily as the H_2S filtrate of $FeCl_3$. Rats A 4, A 5, and A 6 received 0.5 mg. of Fe daily as electrolytic $FeCl_3$.

TABLE III

Prevention and Cure of Nutritional Anemia with Iron

Rats for the prevention study and iron for the curative study were furnished by Dr. W. E. Krauss.

Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery		Diet of whole milk + 0.5 mg. Fe
	Before	After	Before	After	Before	After	Hb	R.b.c.	
							Prevention study		
	gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*	
B 1	52	182	10.0	14.3	4.2	8.8	7	6	Rats did *not* develop
B 2	50	134	10.5	13.5	5.1	7.4	7	6	anemia on our Fe solu-
B 3	50	143	9.1	14.6	4.3	7.4	7	6	tion
							Curative study		
B 4	77	143	2.9	14.2	2.7	8.8	6	5	Rats were cured of their
B 5	83	140	2.6	13.3	2.2	8.6	6	6	anemia by 0.25 mg. Fe
B 6	88	138	3.1	13.5	2.1	8.8	6	6	daily, supplied by Dr.
B 7	52	129	2.6	14.7	2.6	8.9	6	6	Krauss. (All animals
B 8	32	173	3.2	16.6	2.9	7.4	6	3	kept in large glass
B 9	36	128	3.6	16.6	3.1	7.3	6	3	cages).

These investigators were unable to obtain hemoglobin recovery on Fe alone. As will be noted in Tables III and IV their Fe solutions in our hands both protected normal young rats from developing anemia on a whole milk diet and also gave complete blood regeneration in anemic young rats in 5 to 6 weeks.

It seemed desirable in the course of this work to inquire into the etiology of nutritional anemia in the rat. There can be little doubt that it is due to an Fe starvation (as milk contains very

TABLE IV

Prevention and Cure of Nutritional Anemia with Iron

The iron solution containing 20 mg. of Fe as $FeCl_3$ per cc. was furnished by Dr. C. A. Elvehjem.

Rat No.	Body weight		Hb per 100 cc.		R. b.c. per c.mm.		Time for recovery		Remarks
	Before	After	Before	After	Before	After	Hb	R.b.c.	
Prevention study									
	gm.	*gm.*	*gm.*	*gm.*	*mil-lions*	*mil-lions*	*wks.*	*wks.*	
C 1	35	150	9.6	12.5	3.8	8.1	6	6	Whole milk diet. Onset
C 2	33	135	8.9	16.6	4.2	7.9	6	6	of anemia prevented by 0.5 mg. Fe daily
Curative study									
C 3	43	133	3.7	15.7	1.6	7.2	4	4	Whole milk diet and 0.25
C 4	47	121	3.3	12.5	2.0	7.2	4	4	mg. Fe daily
C 5	54	126	3.0	14.4	2.8	7.8	4	4	
C 6	49	133	4.1	14.7	2.1	7.6	4	4	
C 7	51	127	3.1	10.9	1.6	8.4	4	4	

little of this element) during the period of growth from weaning to about 100 gm. of body weight. When this Fe deficiency is made good, regeneration of the blood is rapid and values above normal are sometimes obtained. To test out this hypothesis we conducted the following experiment.

Twenty-three rats, of 90 to 110 gm. body weight, raised on our stock diet, were placed on whole milk alone for a period of 4 months. The average weight of the group reached a plateau of about 170 gm., and the hemoglobin concentration and erythrocyte

counts were normal in every case.   When rats attain a body weight
of about 100 gm. with a normal hemoglobin content, it is impossible
to render them anemic by feeding on milk alone, since their needs for
Fe are apparently not as great as during early growth.   After this
experiment was completed a 200 mg. dried yeast tablet was given
daily to each rat, when increased growth resulted.   It would thus
appear that the milk did not contain enough of the vitamin B
complex for the further growth of the animals.   The milk, how-
ever, supplied enough of these factors for our anemic animals,
since they had all recovered from anemia by the time they had
reached about 100 to 130 gm. body weight.   Many of our rats
after recovery from the anemia were allowed to have milk alone
for some weeks, and no recurrence of the anemic condition was
observed.

Several other incidents were observed in the experiments which
seem worth recording.   A good many cases of diarrhea occurred
during the development of the anemia, although in a large number
of the rats it disappeared quickly.   After treatment with the
various supplements, it disappeared completely, and no deaths
resulted.   A large number of pied rats were used and the milk
diet tended to whiten somewhat the black pigment in the animal's
hair, which always became black again after the animal had been
placed on the stock diet for some time.

*Necessary Precautions in the Conduct of Experiments on Nutri-
tional Anemia of the Rat*

There are a number of precautions which we have followed in
the present studies and which we believe should be considered
in the conduct of experiments on the nutritional anemia of the rat.

1. Quantitative Intake of Supplements*—The supplement (or
supplements) is given in concentrated form (usually 5 drops of
Fe solution, equivalent to 0.25 mg.) in about 1 cc. of cream,
usually at 10 a. m.   This is quantitatively consumed usually by
10.30, at which time more milk is given.   Later in the day more
milk is given, but very little of it is left to sour in the cups.

2. Quantitative Intake of Milk—Under the above conditions
the animals were supplied with fresh cold milk, three or four
times daily.   Knowledge of the amount of milk consumed is often
very helpful.   The milk consumption of our rats has usually been
excellent.

3. Growth—Good growth was practically always obtained. There was little or no blood regeneration in the absence of good growth, but growth may take place without much hemoglobin regeneration (see Paper VI of this series).

4. Hemoglobin and Red Blood Cells—Data on the rise of hemoglobin alone do not give the complete picture of blood regeneration. Many erythropoietic stimulants seem to act first on red blood cell formation. Earlier evidence of stimulation may, therefore, be obtained with the erythrocyte count, and quicker still with the reticulocyte count (see Paper V of this series).

5. Number of Animals Used—The rate of hemoglobin recovery varies in different animals. A sufficient number of rats should be used definitely to establish the time of recovery.

6. Initial Hemoglobin Level—Different rates of recovery will be obtained unless the initial level of hemoglobin is about the same.

DISCUSSION

The view that inorganic Fe cannot be utilized by anemic young rats for blood regeneration is not supported by the experiments recorded in this paper. Recovery of hemoglobin in 6 weeks was obtained when the milk was supplemented with as little as 0.25 mg. of Fe alone daily, while 2.0 mg. of Fe appeared to give a more rapid recovery than any combination of 0.5 mg. of Fe with small additions of other elements.

These findings are quite in agreement with the observations of Mitchell and Schmidt (12), Drabkin and Waggoner (19), and Keil and Nelson (20) on the rat, and Whipple and Robscheit-Robbins (6), Riecker (21), Riecker and Winters (22), and Steiger (23) on the dog, but in disagreement with the observations of Hart, Steenbock, and their coworkers (9, 11) who failed to obtain any effect with the use of pure iron in the nutritional anemia of the rat. Their experiments have apparently received confirmation at the hands of Lewis, Weichselbaum, and McGhee (24), Krauss (25), and Underhill, Orten, and Lewis (26), who obtained negative results with iron alone.

The experiments of Hart, Steenbock, and their coworkers (10) are clean cut and convincing. They maintain that Fe alone is valueless, and must be supplemented by Cu. Our experiments were undertaken with the view that Cu could not hold this unique

rôle. Although we therefore expected to find that some other elements supplemented Fe, such as As, Mn, and Ge (see Paper II of this series), we were quite unprepared to obtain recoveries with Fe alone. It has seemed difficult to reconcile or explain these divergent findings regarding Fe in the nutritional anemia of the rat.

Hart and Steenbock (8, 11) have criticized our experiments (15) and those of Mitchell and Schmidt (12) on the basis that the Fe employed was contaminated with Cu. For reasons given below we do not believe that the differences observed can be explained on this basis. As pointed out under the methods, experiments have been carried out with Fe obtained from six different sources, and in many of the experiments this has been further purified. Identical results as regards hemoglobin regeneration have been obtained with all the preparations. In addition, experiments on six rats were run independently by Dr. F. C. Bing in glass cages to exclude any contact with metal, electrolytic Fe and the H_2S filtrate from the same being employed. The results (Table II) were identical with those reported above. In later experiments identical results were obtained with Fe, kindly supplied by Dr. Elvehjem and Dr. Krauss (see Tables III and IV).

The smallest amount of Cu found by Waddell, Steenbock, Elvehjem, and Hart (27) to give a supplementing action when added to 0.5 mg. of Fe was 0.0025 mg. of Cu. We were unable to elicit a response from so little Cu (see Paper II of this series), but on this basis Cu would be present in the Fe as an impurity to the extent of 0.5 per cent. We do not believe that it is possible our preparations could have been contaminated with this amount of Cu. It should be further noted that in our own experiments it took 0.025 mg. of Cu to obtain a definite supplementing action, which, if present in the 0.5 mg. of Fe used, would have been equivalent to 5 per cent of impurity; 10 per cent in the 0.25 mg. of Fe employed in some of the experiments.

It seemed to us that a much more probable source of difficulty is the milk employed. Owing to the fact that the milk which we had been using came in contact with metal, we arranged to have cows milked into an earthenware jar, and the milk bottled and sent at once to the milk station. 60 experiments were carried out with raw milk obtained in this way, without modifying the results ob-

tained.   From this observation it did not seem probable that our original milk had been altered by its short contact with metal. From a practical standpoint the milk we have used should compare favorably with the very highest grade of (raw) milk distributed in urban centers for use in infant nutrition.   It must be admitted, however, that from this point of view Hart and Steenbock and Krauss have been in a much more fortunate position than ourselves owing to their access to a better known source of milk. Granting the possibility that the results we have obtained with pure Fe may have been influenced by our milk supply, we believe that our experiments are comparable with the conditions which would ordinarily obtain in human nutrition.

It is of interest in this connection that Supplee, 'Dow, Flanigan, and Kahlenberg (28), studying nutritional anemia in rats, found that milk in the process of drying takes up Fe from the container used, and that this Fe can prevent or cure the anemic condition of their animals, as the case may be.   The Cu content of both the dry milk and fluid milk from which it was prepared was the same. A somewhat similar observation has recently been made by McGhee (29) who has noted that when milk is left in contact with certain metals there is sufficient solution to supply the requirements for rapid regeneration of hemoglobin in rat and man.   Cu alone was found to be effective, but less so than when accompanied by other metals, especially Fe.

The amount of milk consumed daily by Hart and Steenbock's rats was not given, but on one occasion they assumed (10) that it amounted to about 35 cc. for a 50 to 75 gm. rat, which on the basis of milk analyses made in their laboratory (17) would contain about 0.005 mg. of Cu, while their lowest effective dose of added Cu was found to be 0.0025 mg.   Thus according to their own data, the 35 cc. of the milk they employed contained about twice the amount of added Cu they found necessary for regeneration, and it is probable that rats which recovered ultimately consumed twice this amount of milk.   Older analyses with the xanthate method would give 0.015 mg. of Cu in 35 cc. of milk, and Supplee and coworkers (28) maintain that this is the most satisfactory method.   The Cu content of milk given by various workers with several different methods varies from 0.15 to 0.5 mg. per liter.   According to the recent analyses of Drabkin and Waggoner (19), 35 cc. of Walker-

Gordon certified milk would contain 0.011 mg. of Cu. In our experiments, recorded in Paper II of this series, 0.025 mg. of Cu was found to be the lowest effective dose of added Cu. The Cu content of our milk was estimated by the method of Elvehjem and Lindow (17) and found to be 0.44 mg. per liter. Thus, in 75 cc. of milk daily there was being given 0.033 mg. of Cu, an amount, if anything, larger than our lowest effective dose of added Cu. For this reason the possibility cannot be denied that the traces of Cu or other inorganic elements in the milk we employed may have exerted some supplementing action on the added iron. However, it seems quite illogical to us that Waddell, Steenbock, and Hart (11) should attribute the hemoglobin regeneration in our rats to the presence of traces of Cu in our Fe, when according to their own analyses the milk must have contained a far greater content of Cu than could have been present in the iron as an impurity.

In working with chicks Elvehjem and Hart (30) found excellent recoveries with $FeSO_4$ made from iron wire, but attribute the results to the Cu content of the Fe solution used. McGowan and Crichton (31, 32) were the first to recognize the anemia of suckling pigs as an Fe deficiency. With these animals the Wisconsin investigators (33) found that it was unnecessary to use Cu salts with $FeCl_3$, yet they recommend the administration of Cu, or impure Fe salts containing it. They explain their different findings on the pig and rat by saying that the Cu requirements of the former are not as great as those of the latter.

It is not believed that the young rats employed in the present experiments were able to store up Fe or Cu from their mother's milk. Elvehjem, Herrin, and Hart (34) have shown in the goat and cow that large additions of these metals to the mother's diet will not prevent the onset of anemia in the young, nor will it increase the Fe and Cu content of the mother's milk. It might be noted, however, that our stock rats all received fresh lettuce, which is rich in minerals, three times per week. Practically all of the young animals developed anemia regardless of their mother's diet and for this reason it would not seem probable that the recovery was due to any "reserves" of these metals as postulated by Titus and Hughes (35).

Our observations leave no doubt that inorganic iron salts, *e.g.* $FeCl_3$, can be utilized in hemoglobin building. In regard to the

question of ferrous and ferric salts, Starkenstein and Weden (36, 37) claim that the therapeutic action of Fe is manifested only in the ferrous compounds and that simple ferric compounds introduced into the organism from the outside are always inactive. According to Sollmann (38) all forms of Fe can be assimilated. Our results and those of others would seem to support this view, since we fed both the ferrous (H_2S filtrate) and ferric Fe and observed an excellent effect upon blood regeneration with both salts.

Our statement that nutritional anemia of young rats on whole milk diets is due to an Fe deficiency is in harmony with the view of Hill (39) that nutritional anemia in infancy is a deficiency disease.   He states:

"We consider this type of anemia to be a definite deficiency disease, comparable to rickets and scurvy, depending upon a lack of iron and possibly pigment in the diet, ... it is possible to produce a high degree of anemia in young growing animals.   It is not possible to do this in adult animals, however, nor do we see anemia in conditions of starvation, ... This means that growth is necessary to produce the type of anemia we are discussing, and the more rapid the growth, the more rapid the development of the anemia.   In this nutritional anemia is analogous to rickets."

From a review of the recent clinical literature it is evident that a number of investigators have been stimulated by the recent experimental studies on animals.   With the point of view that a number of forms of secondary anemia are due primarily to an Fe deficiency, they have been able to obtain excellent results from the administration of Fe, although in some cases the Fe has been supplemented by liver feeding.   Fe and liver together appear to be better than either alone.   The following studies might be mentioned: MacKay (40) on anemic children, Keefer and Yang (41) on various conditions of secondary anemia in China, and Galloway (42) on the physiological anemia of pregnancy (employed As with Fe).

SUMMARY AND CONCLUSIONS

Young rats rendered anemic by feeding whole milk for 6 weeks after weaning were given doses of Fe, electrolytic Fe, and the H_2S filtrates from the same, from 0.08 to 2.5 mg. daily in the form of the chloride.   The results obtained would seem to justify the following conclusions.

1. 0.25 mg. of Fe is the smallest unsupplemented daily dose of this element to give average hemoglobin recovery in 6 weeks and erythrocyte recovery in 4 weeks when added to a whole milk diet. This dose probably represents the minimal daily Fe requirement of the growing young rat suffering from nutritional anemia.

2. 95 anemic young rats were cured by the administration of 0.5 mg. of Fe daily, with average hemoglobin recovery in 6 weeks and erythrocyte recovery in 3.8 weeks.

3. 2 mg. of Fe daily were the most effective dose, average recovery of both cells and hemoglobin taking place in 1.8 weeks. 2.5 mg. were no more effective than 2 mg.

4. Nutritional anemia in young rats fed on whole milk diets is an inorganic Fe deficiency disease, from the time of weaning until about 100 gm. body weight is reached. Since inorganic Fe, *per se*, prevents and cures this condition, it seems unnecessary to assume that any other factors in addition to Fe, either inorganic or organic, are necessary for hemoglobin formation in this particular type of anemia, although traces of many other elements do have a supplementing (catalytic) action on small amounts of Fe.

BIBLIOGRAPHY

1. Hall, W. S., *Arch. Anat. u. Physiol., Physiol. Abt.*, **18**, 455 (1894).
2. Meyer, E., *Ergebn. Physiol., 1 Abt.*, **5**, 698 (1906).
3. Robscheit-Robbins, F. S., *Physiol. Rev.*, **9**, 666 (1929).
4. Elden, C. A., Sperry, W. M., Robscheit-Robbins, F. S., and Whipple, G. H., *J. Biol. Chem.*, **79**, 577 (1928).
5. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, **72**, 419 (1925).
6. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, **92**, 362 (1929).
7. Hart, E. B., Steenbock, H., Elvehjem, C. A., and Waddell, J., *J. Biol. Chem.*, **65**, 67 (1925).
8. Hart, E. B., Elvehjem, C. A., Waddell, J., and Herrin, R. C., *J. Biol. Chem.*, **72**, 299 (1927).
9. Waddell, J., Elvehjem, C. A., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, **77**, 777 (1928).
10. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., *J. Biol Chem.*, **77**, 797 (1928).
11. Waddell, J., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, **83**, 243 (1929).
12. Mitchell, H. S., and Schmidt, L., *J. Biol. Chem.*, **70**, 471 (1926).
13. Mitchell, H. S., and Vaughn, M., *J. Biol. Chem.*, **75**, 123 (1927).

14. Mitchell, H. S., and Miller, L., *J. Biol. Chem.*, **85**, 355 (1929–30).
15. Beard, H. H., and Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, **26**, 510 (1929).
16. Myers, V. C., and Beard, H. H., *J. Am. Med. Assn.*, **93**, 1210 (1929).
17. Elvehjem, C. A., and Lindow, C. W., *J. Biol. Chem.*, **81**, 435 (1929).
18. Bing, F. C., and Baker, R. W., *J. Biol. Chem.*, **92**, 589 (1931).
19. Drabkin, D. L., and Waggoner, C. S., *J. Biol. Chem.*, **89**, 51 (1930).
20. Keil, H., and Nelson, V. E., *Proc. Soc. Exp. Biol. and Med.*, **28**, 392 (1931).
21. Riecker, H. H., *J. Clin. Inv.*, **5**, 141 (1927).
22. Riecker, H. H., and Winters, M. E., *Am. J. Physiol.*, **92**, 196 (1930).
23. Steiger, G., *Arch. exp. Path. u. Pharmakol.*, **137**, 269 (1928).
24. Lewis, G. T., Weichselbaum, T. E., and McGhee, J. L., *Proc. Soc. Exp. Biol. and Med.*, **27**, 329 (1929–30).
25. Krauss, W. E., *J. Biol. Chem.*, **90**, 267 (1931).
26. Underhill, F. A., Orten, J. M., and Lewis, R. C., *J. Biol. Chem.*, **91**, 13 (1931).
27. Waddell, J., Steenbock, H., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **83**, 251 (1929).
28. Supplee, G. C., Dow, O. D., Flanigan, G. E., and Kahlenberg, O. J., *J. Nutr.*, **2**, 451 (1930).
29. McGhee, J. L., *Science*, **73**, 347 (1931).
30. Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **84**, 131 (1929).
31. McGowan, J. P., and Crichton, A., *Biochem. J.*, **17**, 204 (1923).
32. McGowan, J. P., and Crichton, A., *Biochem. J.*, **18**, 265 (1924).
33. Hart, E. B., Elvehjem, C. A., Steenbock, H., Bohstedt, G., and Fargo, J. M., *J. Nutr.*, **2**, 277 (1929).
34. Elvehjem, C. A., Herrin, R. C., and Hart, E. B., *J. Biol. Chem.*, **71**, 255 (1926–27).
35. Titus, R. W., and Hughes, J. S., *J. Biol. Chem.*, **83**, 463 (1929).
36. Starkenstein, E., and Weden, H., *Klin. Woch.*, **7**, 220 (1928).
37. Starkenstein, E., and Weden, H., *Arch. exp. Path. u. Pharmakol.*, **150**, 354 (1930).
38. Sollmann, T., A manual of pharmacology, Philadelphia and London, 3rd edition, 999 (1926).
39. Hill, L. W., *New England J. Med.*, **201**, 261 (1929).
40. MacKay, H. M. M., *Proc. Roy. Soc. Med., Dis. Child. Sect.*, **22**, 29 (1929).
41. Keefer, C. S., and Yang, C. S., *Nat. Med. J. China*, **15**, 701, 721, 731, 743 (1929).
42. Galloway, C. E., *J. Am. Med. Assn.*, **93**, 1695 (1929).

STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

II. INFLUENCE OF IRON PLUS SUPPLEMENTS OF OTHER IN-ORGANIC ELEMENTS UPON BLOOD REGENERATION

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In the clinical treatment of certain forms of secondary anemia a number of elements, in addition to iron, have been employed, sometimes as a supplement to iron and sometimes alone. The earlier literature of elements other than iron was reviewed by Wolf (1) in 1898 and by Loewi (2) in 1907. They include in their list of elements which are reputed to be of therapeutic value in blood formation: arsenic, manganese, mercury, copper, and zinc. References are given to papers by several investigators (Cervello and Barbini, Cervello, Scarpinato, and Giudiceandrea) who noted increased blood formation after the use of small doses of copper in human anemia, and in experiments with chickens. In his text-book on practical therapeutics, Hare (3) mentions arsenic, manganese, copper, and mercury, while several years ago germanium received some attention due to the work of Hammett, Nowrey, and Müller (4).

The recent work of Hart, Steenbock, and their coworkers (5) on the supplementing action of Cu to Fe in bringing about blood regeneration in rats suffering from nutritional anemia has emphasized anew the importance of other elements in addition to Fe, particularly Cu, in the treatment of anemia. Their work has given considerable impetus to this line of investigation. The supplementing action of Cu emphasized by Hart and Steenbock has been uniformly confirmed by a number of investigators.

Although much impressed by the experiments of Hart, Steenbock, and their coworkers, it did not seem to us that Cu could play the unique rôle in hemoglobin metabolism which their conclusions

implied.   Experimental evidence for this view was soon forth-
coming in the researches of Titus, Cave, and Hughes (6) and Goer-
ner (7) with respect to Mn, which these workers found to possess
much the same stimulating property ascribed to Cu by Hart and
Steenbock.

In spite of experimental evidence that both Mn and Cu possess
this stimulating action with Fe on hemoglobin formation, the
Wisconsin investigators have maintained in several publications
that Cu has a specific and essential action as a supplement to Fe
in hemoglobin building.   Somewhat the same conclusion has
been reached by Krauss (8) and by Underhill, Orten, and Lewis
(9) who were unable to obtain recovery with Fe alone or a supple-
menting action with elements other than Cu in rat experiments.
Titus, Cave, and Hughes (6), on the other hand, found that Cu
and Mn supplemented Fe better than either Cu or Mn alone,
while Mitchell and Miller (10) feel that not copper alone but a
group of elements is active in hemoglobin building.

Experiments carried out on animals other than the rat have
not been so definite in showing a supplementing action of Cu to Fe.
Elden, Sperry, Robscheit-Robbins, and Whipple (11) found that
the iron salt effect was much more notable than the copper effect
in their experiments on hemorrhagic anemia in dogs.   Elvehjem
and Hart (12) were able to produce anemia in day old chicks on a
diet of milk, polished rice, $CaCO_3$, and NaCl, and cure the same
by the addition of pure $FeSO_4$ or $FeCl_3$, although they ascribe
this to the presence of small amounts of Cu in the basal ration.
In experiments on the pig, Hart, Elvehjem, and Steenbock (13)
go even further and admit that pure $FeCl_3$ is effective in curing the
nutritional anemia.

During the past 3 years we have made a study of the Fe-supple-
menting action of a number of elements in the nutritional anemia
of the rat, the results for sixteen of which are presented in this
paper.   Preliminary reports of our observations on Fe and Fe
plus Cu, Ni, Ge, Mn, and Co were presented some time ago (14–
16).   Final report of this work has been delayed until now owing
to the many perplexing questions which have come up during the
course of these experiments.   We have been fortunate in having
the counsel of Dr. Torald Sollmann of the Department of Pharma-
cology during this investigation.

Experimental Methods

The experimental procedures were the same as outlined in Paper I of this series. As supplements we used c.p. copper sulfate (Merck), the chlorides of magnesium and zinc (Baker Analyzed Reagents), nickel and cobalt (Mallinckrodt), rubidium, chromium, selenium, vanadium, and titanium (Tested Purity, Eimer and Amend), germanium dioxide (New Jersey Zinc Company), arsenic acid, c.p. (Baker), and the bichloride of mercury (Merck).

All salts were dissolved in water, or a very small amount of concentrated HCl, then made up to contain 1 mg. of the element per cc. Finely powdered V and Se were fed in suspension in water. The concentrated HCl and HNO_3 used in the preparation of the $FeCl_3$ were Mallinckrodt's reagents.

Results

Copper—The results obtained with this element are given in Table I. Doses from 0.00025 to 0.01 mg. daily with 0.5 mg. of Fe did not give recovery in less than 6 weeks, which is the time for the response on this amount of Fe alone (see Table VII). Thus doses below 0.025 mg. daily were without significant effect upon hemoglobin regeneration. Doses from 0.025 to 0.1 mg. daily all gave the same reaction as did 0.05 mg., namely, 2 to 3 weeks. It would appear that in these experiments 0.025 mg. of Cu was the lowest effective dose of added Cu.

Nickel—This element was fed in daily doses from 0.013 to 0.05 mg. with 0.5 mg. of Fe (Table II). There was no better recovery than 6 weeks until 0.05 mg. of Ni daily was given. From these experiments it would appear that 0.05 mg. is the smallest effective dose of added Ni.

Arsenic—This element was fed in daily doses from 0.002 to 0.01 mg. No effect was observed until the latter dose was employed (Table II). Hence 0.01 mg. of As with 0.5 mg. of Fe is the smallest daily dose of this element that is effective in this connection.

Germanium—This element in the form of the dioxide was effective in all doses from 0.05 to 0.5 mg. daily with Fe (Table III). It is possible that doses lower than 0.05 mg. might also

give this effect, but this was the smallest dose employed in these experiments.   Sodium germanate was prepared from the dioxide.

TABLE I

Influence of Cu Added to Milk-Fe Diet upon Blood Regeneration in Nutritional Anemia

	Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b.c.
		gm.	*gm.*	*gm.*	*gm.*	*mil-lions*	*mil-lions*	*wks.*	*wks.*
Whole milk + 0.5 mg.	15	113	151	6.2	13.8	5.3	9.3	2	2
Fe and 0.05 mg. Cu	16	98	126	4.8	14.0	4.8	9.3	2	1
daily	32	90	104	7.1	14.0	3.8	9.0	2	1
	39	100	115	5.2	15.5	4.6	8.4	2	2
	40	88	122	5.1	13.3	3.3	8.4	2	2
	41	90	115	7.1	14.8	4.9	8.1	3	3
	42	81	106	4.8	13.6	4.1	8.2	3	3
	43	98	110	5.4	14.7	5.6	8.0	3	3
	44	71	110	5.3	13.1	4.8	8.4	3	3
	45	84	113	5.8	14.4	3.4	7.7	2	2
	46	75	94	5.2	13.4	3.5	8.1	2	2
Average..................		89.8	115	5.6	14.1	4.4	8.4	2.4	2.2
Whole milk + 0.5 mg.	17	72	105	2.3	12.5	2.3	7.5	6	3
Fe. Rats 17–24 re-	18	60	115	3.0	12.5	1.9	7.6	6	3
ceived 0.0025 to 0.01	19	114	143	2.0	14.2	1.5	8.5	6	2
mg. Cu daily while	20	74	105	3.0	16.0	1.4	8.1	5	2
Rats 25–30 received	21	109	205	2.5	13.5	1.5	7.8	3	2
0.025 to 0.1 mg. Cu	22	78	121	2.5	13.3	1.3	7.7	5	5
	23	58	125	3.0	13.3	1.7	7.7	5	6
	24	108	130	3.0	13.2	1.5	7.5	6	5
	25	76	125	2.5	14.3	1.9	7.6	2	3
	26	59	102	3.0	15.6	1.9	7.7	2	2
	27	97	157	3.0	13.6	1.9	8.1	3	2
	28	77	160	2.0	14.6	1.5	7.0	3	2
	29	117	157	2.3	13.3	1.8	10.8	2	3
	30	102	138	2.5	14.1	2.0	7.1	3	3
Average.........		85.5	134.8	2.6	13.8	1.7	7.9	4.1	3.1

Doses of 0.05 to 0.4 mg. were fed daily with Fe, but recovery was not obtained until the latter dose was given.   This is contrary to

the observations of Nowrey (17), who found that the germanate was more effective than the dioxide.

TABLE II

Influence of Ni and As Added to Milk-Fe Diet upon Blood Regeneration in Nutritional Anemia

	Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b.c.
		gm.	gm.	gm.	gm.	millions	millions	wks.	wks.
Whole milk + 0.5 mg.	93	63	109	7.6	15.4	3.4	8.4	3	2
Fe and 0.05 mg. Ni	94	52	91	6.1	13.3	4.6	9.6	3	1
daily	95	63	91	5.5	13.7	4.1	8.9	1	2
	96	59	97	5.5	13.5	2.3	9.0	3	2
	128	61	92	5.0	16.4	2.8	8.6	3	1
	129	60	96	5.0	13.3	4.0	7.8	3	2
	130	53	99	7.1	14.3	3.6	8.7	3	2
	131	54	98	5.9	14.1	1.8	8.1	3	2
	132	58	75	6.6	14.3	3.1	8.2	3	2
	140	50	78	4.2	14.7	2.4	8.1	3	3
	141	53	91	4.6	14.3	2.5	8.7	3	3
	142	45	86	5.0	13.5	3.5	8.9	3	3
	144	63	106	4.4	14.2	2.0	8.3	3	2
Average................		56.5	93.0	5.6	14.2	3.1	8.6	2.8	2.1
Whole milk + 0.5 mg.	133	55	84	4.8	13.6	2.8	7.9	3	2
Fe and 0.01 mg. As	134	62	93	4.1	14.1	3.1	8.2	3	2
daily	135	54	96	4.6	14.0	3.9	8.9	3	2
	136	57	98	5.7	13.3	3.0	8.6	3	1
	137	61	104	5.5	14.3	3.2	9.6	3	1
	138	51	86	4.6	13.3	3.2	8.0	3	3
	139	50	92	4.2	14.0	2.6	8.9	3	3
	145	62	92	4.6	13.6	2.6	8.2	3	2
	146	58	96	4.4	14.1	2.5	7.9	3	2
	151	61	82	4.6	14.2	3.4	9.8	3	2
	152	58	80	3.8	13.2	4.1	8.8	3	2
	153	66	86	4.4	13.6	3.5	8.4	3	2
Average................		58	90	4.6	13.8	3.2	8.6	3	2

Manganese—This element was fed in daily doses of 0.025 to 0.5 mg. (Table IV). The lowest optimum dose is seen to be 0.1

TABLE III

Influence of GeO₂ and Na₂GeO₃ Added to Milk-Fe Diet upon Blood Regeneration in Nutritional Anemia

	Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b.c.
		gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*
Whole milk + 0.5 mg.	122	61	106	6.6	12.5	4.6	8.4	3	2
Fe and 0.05 mg. Ge	123	58	96	4.3	14.3	1.8	8.2	3	2
as GeO₂ daily	124	61	96	4.3	13.1	1.9	8.4	3	2
Whole milk + 0.5 mg.	125	61	96	7.1	15.9	3.6	8.7	3	2
Fe and 0.1 mg. Ge	126	62	97	4.6	13.8	1.7	8.0	3	2
as GeO₂ daily	127	71	109	4.6	13.6	1.6	7.9	3	2
	157	61	97	4.2	13.7	3.2	8.6	2	2
	158	63	105	4.8	14.0	2.9	8.6	3	3
	159	48	81	4.4	15.3	2.4	8.6	3	3
	160	55	92	4.3	15.3	3.2	8.0	3	2
Whole milk + 0.5 mg.	14	107	120	7.1	13.2	3.6	8.2	3	2
Fe and 0.5 mg. Ge	28	91	108	5.7	14.8	5.5	8.9	2	2
as GeO₂ daily	29	78	97	5.2	16.0	3.4	9.4	2	2
	30	92	118	5.4	16.7	4.6	8.6	2	1
	31	84	105	5.1	13.3	3.1	8.1	2	2
	60	138	166	5.4	14.2	3.0	8.9	3	2
	61	112	129	7.7	13.4	5.1	8.2	3	3
	63	89	116	4.7	13.6	3.9	8.4	3	3
	64	93	117	6.8	13.6	3.3	8.1	2	2
	118	58	106	4.8	14.7	2.5	8.8	3	2
	119	58	96	4.6	13.6	3.0	8.2	3	2
	120	61	96	5.4	14.3	2.7	7.9	3	2
	121	60	86	4.3	13.3	2.9	7.6	3	2
Average..............		74.9	105.9	5.3	14.2	3.2	8.4	2.7	2.1
Whole milk + 0.5 mg.	87	60	119	2.9	13.6	2.4	8.3	3	2
Fe and 0.4 mg. Ge	88	40	102	3.6	13.1	1.8	8.0	3	3
as Na₂GeO₃ daily	177	60	111	3.0	13.6	2.4	8.9	3	2
	178	67	110	2.8	14.1	2.8	8.1	3	2
	179	71	108	2.6	12.4	2.5	8.4	3	3
	180	66	117	3.1	13.6	2.8	7.9	3	2
Average..............		60.6	111.1	3.0	13.4	2.5	8.3	3.0	2.3

TABLE IV

Influence of Mn, Ti, and Zn Added to Milk-Fe Diet upon Blood Regeneration in Nutritional Anemia

	Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b.c.
		gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*
Whole milk + 0.5 mg. Fe and 0.1 mg. Mn daily	154	58	85	4.6	13.6	3.0	8.4	3	2
	155	66	88	3.5	13.6	3.5	8.8	3	2
	156	54	79	4.1	14.2	3.6	7.8	3	2
	161	45	72	4.3	14.3	2.7	12.0	2	3
	162	68	83	4.3	16.6	2.4	10.1	3	3
	163	61	83	4.1	15.6	2.4	8.0	3	2
	164	58	87	6.0	14.1	2.8	8.1	3	2
	165	63	90	4.6	14.6	2.4	7.9	3	2
	166	56	85	4.3	13.7	2.3	8.2	3	2
	167	72	105	4.0	13.1	3.2	8.1	2	2
	174	65	90	3.9	13.6	3.8	8.0	3	2
Average..............		60	86	4.3	14.3	2.9	8.7	2.8	2.2
Whole milk + 0.5 mg. Fe and 0.1 mg. Ti daily	272	61	135	4.8	13.3	1.8	7.8	4	3
	273	66	156	5.8	13.3	1.5	8.2	3	2
	274	58	139	6.0	14.0	3.5	8.1	3	3
	275	58	76	5.3	14.3	1.5	7.7	3	4
	276	61	105	4.7	13.3	3.9	8.5	3	2
	277	64	91	3.0	13.3	1.3	8.2	2	2
	279	55	120	3.0	13.3	1.0	8.4	4	3
	280	56	102	7.6	13.3	3.8	7.6	3	3
Average..............		59.9	115.5	5.0	13.5	2.3	8.0	3.1	2.7
Whole milk + 0.5 mg. Fe and 0.1 mg. Zn daily	197	72	117	5.1	16.6	4.8	9.8	3	2
	198	62	97	4.8	14.7	3.8	9.7	3	2
	220	115	143	4.5	13.6	3.6	8.3	3	2
	221	109	144	3.6	13.2	4.1	8.1	3	2
	270	85	96	5.8	14.7	3.8	7.9	3	3
	271	92	115	5.5	14.2	2.8	7.6	3	3
	281	58	75	3.0	14.0	1.4	7.9	3	3
	282	65	94	6.1	14.0	3.4	7.6	3	2
Average..............		82.3	110	4.8	14.4	3.5	8.2	3.0	2.4

mg. of Mn with 0.5 mg. of Fe daily. This confirms the results of Titus, Cave, and Hughes (6) and Goerner (7) in this connection.

TABLE V

Influence of Rb, V, Cr, and Se Added to Milk-Fe Diet upon Blood Regeneration in Nutritional Anemia

	Rat No.	Body weight		Hb per 100 cc.		R.b c. per c.mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b.c.
		gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*
Whole milk + 0.5 mg.	288	46	65	4.5	16.8	0.9	7.1	3	2
Fe and 0.05 mg. Rb	290	88	102	7.0	13.3	2.0	8.2	1	2
daily	287	80	110	4.7	13.8	2.2	8.1	2	3
	320	80	122	3.0	13.3	3.0	7.9	4	2
	321	106	118	4.0	13.3	4.1	8.4	4	3
Average................		80	103.4	4.6	14.1	2.4	7.9	2.8	2.4
Whole milk + 0.5 mg.	291	80	89	4.9	16.5	1.1	9.5	3	3
Fe and 0.05 mg. V	292	86	106	5.5	14.3	1.3	7.8	2	2
daily	293	72	85	6.2	16.6	2.2	8.1	3	2
	322	60	97	2.7	13.3	2.7	8.1	4	2
	323	70	114	3.0	13.3	3.1	8.0	4	2
	324	92	121	3.0	13.3	3.1	8.6	3	2
Average................		77	102	4.2	14.6	2.2	8.3	3.2	2.0
Whole milk + 0.5 mg.	294	80	90	5.6	16.8	3.0	7.1	3·	1
Fe and 0.05 mg. Cr	295	96	116	5.9	16.6	4.0	7.3	3	1
daily	296	93	115	6.2	15.4	2.8	7.7	3	2
	325	100	121	2.7	13.2	2.8	7.7	4	2
	326	106	141	3.6	13.2	3.6	7.3	4	2
	327	90	137	2.8	13.3	2.9	8.9	3	2
Average................		94	120	4.5	14.8	3.2	7.7	3.3	1.7
Whole milk + 0.5 mg.	297	58	86	3.0	14.7	0.9	7.4	3	2
Fe and 0.05 mg. Se	298	94	116	4.6	16.8	1.1	7.3	3	2
daily	299	84	99	6.8	13.3	2.1	8.2	1	2
	328	127	166	2.8	13.3	2.9	8.9	3	2
	329	109	172	2.5	13.3	2.6	7.6	3	2
	330	103	152	2.8	13.3	2.8	7.5	3	3
Average................		96	132	3.8	14.1	2.1	7.8	2.7	2.2

Titanium—Only one dose of this element was fed; namely, 0.1 mg. of Ti with 0.5 mg. of Fe (Table IV), and this amount was found to be effective.

Zinc—This element was fed in daily doses from 0.05 to 0.5 mg. The former dose gave only slightly better stimulation than Fe alone, namely 4 weeks for both cells and hemoglobin (Table IV). 0.1 mg. was the smallest dose to give an effect, while 0.5 mg. retarded blood regeneration.

Rubidium, Vanadium, Chromium, and Selenium—These elements were first fed in doses of 0.1 mg. daily. There was no effect better than 6 weeks. However, when 0.05 mg. of each element was fed separately with 0.5 mg. of Fe, recovery was complete in

TABLE VI

Influence of Hg Added to Milk-Fe Diet upon Blood Regeneration in Nutritional Anemia

	Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery	
		Before	After	Before	After	Before	After	Hb	R.b c.
		gm.	*gm.*	*gm.*	*gm.*	*mil-lions*	*mil-lions*	*wks.*	*wks.*
Whole milk + 0.5 mg.	300	85	100	6.4	13.0	1.3	8.0	2	1
Fe and 0.04 to 0.15	301	103	111	6.2	13.3	2.2	7.5	2	2
mg. Hg daily	302	124	132	6.7	13.3	4.1	9.5	2	2
	303	69	87	6.3	14.7	3.8	9.0	2	1
	304	68	83	4.5	13.5	2.1	8.2	2	2
	305	86	95	5.6	16.6	4.0	9.0	2	2
	331	110	152	2.5	13.3	2.5	7.3	4	2
	332	123	150	2.8	13.3	2.8	8.4	4	2
	333	92	131	2.3	13.4	2.3	8.3	4	3
Average.................		95.5	115.7	4.8	13.8	2.8	8.3	2.7	1.9

2 to 3 weeks, and this amount would appear to be the optimum dose of these elements (Table V).

Mercury—Mercury was fed in doses of 0.001 to 0.15 mg. daily, with 0.5 mg. of Fe (Table VI). No effect better than 6 weeks was noted until 0.04 to 0.15 mg. was given, and thus it would appear that 0.04 mg. was the lowest effective dose of this element.

Cobalt—Cobalt was fed in doses from 0.05 to 0.3 mg. daily with 0.5 mg. of Fe. With doses of 0.05 and 0.1 mg. there was an evident initial stimulus, but this was subsequently lost and there was no recovery better than 6 weeks. When doses up to 3 mg. daily were fed, the animals refused to drink the milk. It would

appear from these experiments that Co in daily doses from 0.05 to 0.3 mg. has no sustained stimulating effect on blood regeneration when added to Fe as a supplement.

Magnesium—This element was fed in daily doses from 0.05 to 0.5 mg.   There was no effect upon hemoglobin formation better than 6 weeks.   The larger dose retarded blood regeneration.

Aluminum—Daily doses from 0.2 to 7 mg. of this element were fed to a total of thirty-two anemic rats.   A few of these animals showed quite good responses the 1st week, but 6 weeks were eventually required for complete regeneration in all cases.   It is probable that there was very little absorption, if any, of Al, but judging from these experiments this element when fed by mouth has no supplementing action to Fe in blood regeneration.

It is evident from the results obtained with the above elements that, with the exceptions of Co, Mg, and Al, all possess the power of stimulating blood regeneration when fed daily with 0.5 mg. of Fe, but only in the optimum doses.

The importance and value of making erythrocyte counts in addition to hemoglobin estimations is emphasized in Paper V of this series, and is also brought out in Tables I to VI.   The erythropoietic action often becomes evident first, or is more pronounced in the red blood cells than in the hemoglobin.   It would seem that in most cases there was a greater initial stimulus to red blood cell formation than to hemoglobin production.   It will be noted in Tables I to VI that, although the hemoglobin regeneration was slightly more prompt with Cu than with any other supplement, the red blood cell formation was slightly more prompt with Ni and Ge, and definitely more prompt with As, V, Cr, and Hg than with Cu.

The importance of the dosage of the different elements used in studies of this nature cannot be overemphasized.   In Table VII are given the average results obtained with seventeen groups, totaling 169 rats, when several doses other than the optimum reported above were fed.   In most experiments the reaction with Fe alone was obtained in 6 weeks, and thus with the doses of the supplements employed there was no accelerating influence on blood regeneration.   In some experiments, those with Mg and Zn, for example, hemoglobin recovery was delayed.   Very small doses of Hg gave incomplete recovery in 5 weeks, and the same

was true with 0.1 mg. of Rb, V, Cr, and Se. Mn in doses from
0.02 to 0.4 mg. gave incomplete recovery in 6 weeks, while it will
be recalled that 0.1 mg. daily gave recovery in 2 to 3 weeks.

It is evident from these observations that one is able to hasten,
retard, or obtain the usual Fe reaction in 6 weeks, by simply
varying the daily dose of the element used as a supplement.

TABLE VII

*Effect of Unfavorable Doses of Elements with Fe upon Blood Regeneration,
Except Mg, Co, and Al*

Average values.

No. of animals	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Time for recovery		Addition made to daily diet of whole milk and 0.5 mg. Fe
	Before	After	Before	After	Before	After	Hb	R.b.c.	
	gm.	gm.	gm.	gm.	mil-lions	mil-lions	wks.	wks.	
14	98.0	134.0	3.6	13.7	2.5	8.0	6.3	4.1	0.013 to 0.4 mg. Ni
18	91.3	121.2	3.3	13.4	2.4	8.1	5.7	3.6	0.002 " 0.1 " As
3	68.0	131.0	4.0	14.3	3.0	8.4	10.0	7.0	0.5 mg. Mg
8	86.0	126.0	5.2	11.8	3.4	8.6	5.7	4.6	0.05 and 0.1 mg. Mg
3	70.0	127.0	3.9	14.5	3.3	8.9	10.5	7.7	0.5 mg. Zn
6	98.0	131.0	5.8	16.7	3.5	8.6	4.0	3.2	0.05 mg. Zn
33	89.3	141.9	2.8	13.8	2.3	8.0	5.7	3.5	0.00025 to 0.01 mg. Cu
5	119.0	132.0	6.0	6.9	3.5	5.7	5.0	5.0	0.003 to 0.009 mg. Hg
5	112.0	119.0	5.0	7.9	3.7	6.0	5.4	4.6	0.005 " 0.015 " "
4	97.0	124.0	6.2	8.7	3.6	6.8	6.0	4.7	0.1 mg. Rb
5	113.0	125.0	5.3	7.7	3.0	4.9	5.2	5.0	0.1 " V
5	93.0	114.0	6.2	9.1	3.9	6.2	5.6	5.0	0.1 " Cr
5	109.0	138.0	6.2	10.3	4.0	7.3	5.0	4.6	0.1 " Se
4	80.0	130.0	3.2	13.8	2.2	6.9	6.0	3.0	0.02 to 0.05 mg. Mn
4	76.0	84.0	5.6	6.5	2.7	6.3	6.0	6.0	0.2 to 0.4 mg. Mn
15	76.5	108.0	5.3	12.0	3.0	8.1	5.6	4.0	0.05 to 0.3 mg. Co
32	66.5	111.7	3.8	12.9	3.1	7.8	6.0	4.2	0.2 to 7 mg. Al

*Effect upon Blood Regeneration of Optimal Doses of Mineral Supple-
ments with Doses of Fe Varying from 0.05 to 0.25 Mg.*

Since many elements have a definite catalytic effect on Fe in
stimulating blood regeneration in the nutritional anemia of the
rat, it is of importance to compare the action of these elements
when they are given with doses of Fe below the minimal effective
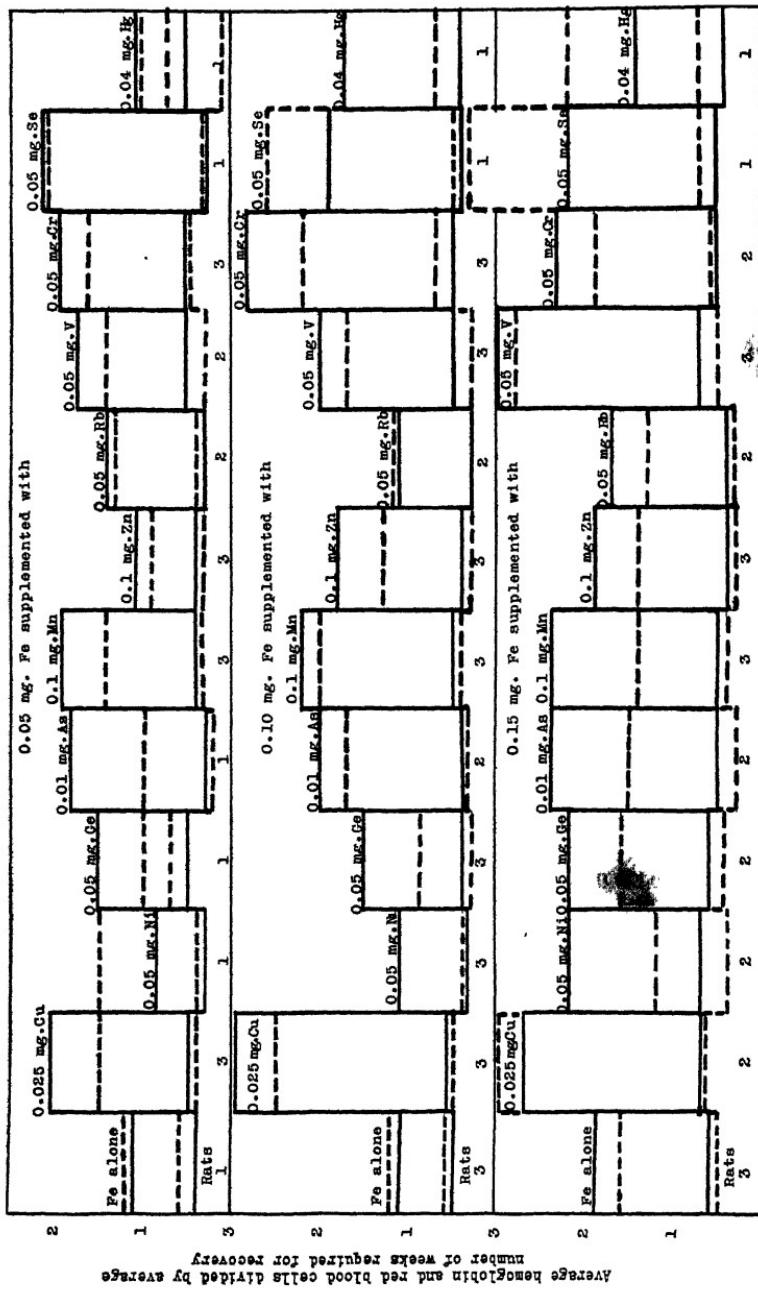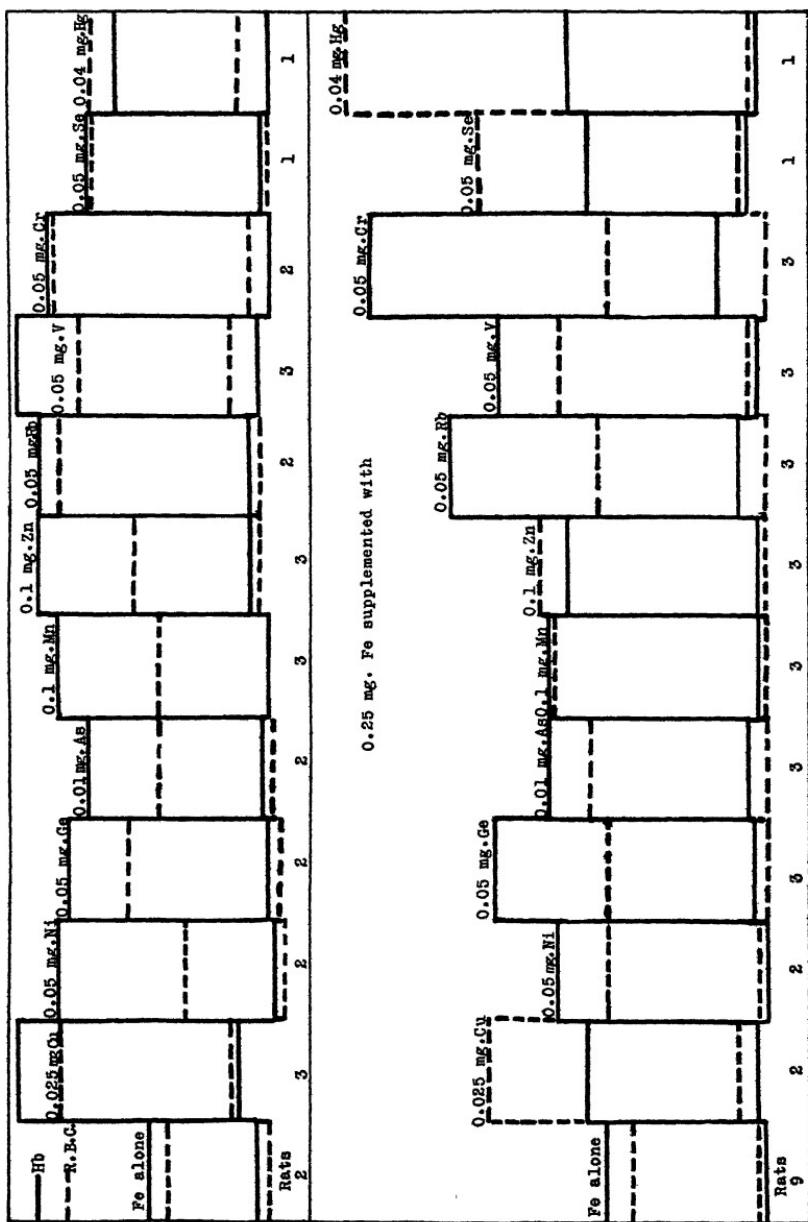dose (0.25 mg. of Fe daily for 6 weeks).

CHART I. Hemoglobin regeneration with 0.05, 0.10, and 0.15 mg. of Fe daily plus inorganic supplements. The solid line, indicates Hb, the dash line, R.b.c. The initial Hb and R.b.c. values, as well as the recovery values, were divided by the weeks required for recovery, and are represented by the lower bars.

Average hemoglobin and red blood cells divided by average number of weeks required for recovery

Five groups of anemic young rats consisting of fifteen animals to the group (with the exception of the Se and Hg groups, in which only one animal was used for each dose of Fe) were fed optimal doses of the supplements with the following doses of Fe: Groups 1 to 5 with 0.05, 0.10, 0.15, 0.20, and 0.25 mg. of Fe daily, respectively.   The comparative data for erythrocyte and hemoglobin regeneration are given in Charts I and II.

Comparisons of hemoglobin recovery shown on Charts I and II may be summarized briefly as follows:

Daily dose of Fe mg.	Hemoglobin regeneration
0.05	Cu > As > Mn > Cr
0.10	Cu > Cr > Mn > As
0.15	V > Cu > Mn > As
0.20	V > Cu > Cr > Ni
0.25	Cr > Rb > Ge > V

On the whole it would appear that Cu was slightly the best Fe supplement in this series of experiments, especially with very small doses of Fe.   It is possible, therefore, so to adjust the dose of Fe and various supplements as to bring out a superiority of Cu over other supplements, and this may offer a partial explanation for the findings of Hart, Steenbock, and their coworkers (5), and others, who have obtained a supplementing action only with Cu. With an incomplete absorption of the larger dose of Fe (0.5 mg.), for example, results such as we have just reported might be obtained.   It is of interest in this connection that Mitchell and Miller (18) found that Cu was most effective with small doses of Fe.

The one rat fed on 0.05 mg. of Fe with 0.05 mg. of Se gave even better results than Cu in this connection, and it is possible that Se may be better than Cu with this small dose of Fe.   Cr gave essentially the same results as Cu when both were fed with 0.1 mg. of Fe.   V replaces Cu with 0.15 and 0.20 mg. of Fe, while Cr was the best element with 0.25 mg.   The differentiation of the elements with Fe is somewhat lost with 0.15 to 0.25 mg. of Fe, since more or less complete recovery was obtained with all the different supplements.

The data on survival are interesting.   If one will subtract the number of animals shown on Charts I and II from three, the number

of animals at the start of the experiment (excepting the observations on Se and Hg), it will give the number which died.   For instance with 0.05 mg. of Fe, all animals on Cu, Mn, Zn, and Cr lived, while two on Rb and V, and one on Ni, Ge, As, and Fe alone died.   This furnishes additional evidence of the stimulating action of several elements with Fe on blood regeneration.

DISCUSSION

The discussion which has arisen over the value of a number of inorganic elements in experimental anemia would appear to be a continuation of the age long controversy which has been waged over their clinical value as therapeutic agents.   The observations reported above are in harmony with the clinical belief that a number of elements serve as erythropoietic stimulants in the treatment of certain forms of secondary anemia.   It is a well known fact that certain therapeutic agents may have almost diametrically opposite effects depending upon the dosage.   Many of the elements which we have shown to possess a supplementing action to Fe in the relief of the nutritional anemia of the rat, are known to produce anemia when the dose is sufficiently large.   Among these may be mentioned arsenic, manganese, mercury, and vanadium. In suitable doses some of these elements have also been shown to produce a polycythemia.   This has been reported with arsenic, manganese, phosphorus, germanium, and cobalt (see Paper IV of this series).

The therapeutic use of arsenic in the treatment of secondary anemia is so well known as to require no comment.   Manganese also appears to have been quite commonly employed for this purpose.   As already pointed out, Titus, Cave, and Hughes (6) and Goerner (7) found that 0.1 mg. of Mn served as an effective supplement to Fe in the nutritional anemia of the rat.   Hg, Zn, and Cu have also been used clinically, although their action in this connection does not seem to be very well known.   The elder Keyes (19) first noted the beneficial effect of Hg in syphilitic anemia, and its use seems to have had quite a vogue in the treatment of secondary anemia.   The literature has been reviewed by Tiffeneau (20).   The stimulating action of Zn on red blood cell and hemoglobin formation in both man and animals has long been recognized (1), and reference has also been made in the introduc-

tion to the early literature on Cu. It would appear that the erythropoietic action of Cu had been recognized 30 years before Hart and Steenbock announced their discovery of its Fe-supplementing action in the nutritional anemia of the rat.

Following the pioneer observations of Hammett, Nowrey, and Müller (4), there have been a number of publications (4, 21–25) supporting their observations that Ge served as a stimulant for red blood cell and hemoglobin production. Dr. Rosalie M. Parr (in a personal communication to the authors) states that she has been able to confirm the erythropoietic action of Ge on the rat and man. It should perhaps be noted that Bailey, Davidson, and Bunting (26), Minot and Sampson (27), and Whipple and Robscheit-Robbins (28) have disagreed with the conclusions of Hammett, Nowrey, and Müller (4) regarding this element. In our hands Ge was quite effective and in fact gave red blood cell counts much above normal in many cases (see Paper IV of this series).

On the basis of the observations recorded in the older literature and the data we have just presented, it seems illogical to us to assume that Cu is a unique supplement to Fe in the formation of hemoglobin. It would appear to be equal if not superior to most other elements in this connection (see Chart I), but its action is not specific. There are a number of elements which act as erythropoietic stimulants.

It is our opinion that in studies of the nutritional anemia of the rat, investigators should not depend solely upon hemoglobin recovery for their criterion of blood regeneration. The initial stimulus of many erythropoietic agents is upon red blood cell formation, rather than upon hemoglobin production. As pointed out, on p. 98 important information may be lost if red blood cell counts are not made.

The nearest approach to agreement with the observations of Hart, Steenbock, and their coworkers we have been able to make is given in Charts I and II. When the amount of Fe added to the milk was cut down to 0.05 and 0.1 mg., Cu was found to supplement the Fe slightly better than any other element. It might be possible, therefore, so to adjust the conditions of experiment as to elicit an effect with Cu without obtaining an appreciable effect with other supplements. Although Cu supplemented 0.05 mg. of Fe better than any other element, it will be observed from an

inspection of Chart I that the supplementing action of Mn was also quite good.

The manner of administering the mineral supplements is, we believe, very important. It has been our custom to give them the first thing in the morning while the animals are hungry, in concentrated form, in a small volume of cream (top of milk). Subsequent milk is not given until the supplements are consumed. This insures their complete consumption at a time when the maximum acidity is probably present in the stomach.

The criticism has been made that the elements which we have used to supplement the Fe may be contaminated with Cu. We do not believe that this requires serious answer because of the very high percentage content of Cu this would require. We might point out that in the case of As, the optimum supplement is one-fifth of the Cu, *i.e.* 0.01 mg. of As in comparison with 0.05 mg. of Cu. With the minimum dose of Cu we have found effective, 0.025 mg. of Cu, this would mean a 250 per cent contamination.

The criticism of our work might be made that our basal diet (milk) contained sufficient Cu to bring about recovery, and that the various Fe supplements which we have employed, simply exercised a catalytic effect upon this small amount of Cu. It does not seem to us that when essentially the same effects were obtained with a number of other inorganic supplements in addition to Cu, their action can be explained so simply. As previously mentioned, some investigators in this field believe that several elements together act better than Cu alone.

There are other agents besides the inorganic elements which have been found favorably to influence blood formation. Thus Furniss (29) found that ultra-violet irradiation increased the hemoglobin and erythrocytes. Osato and Tanaka (30), observed somewhat the same effect in hemorrhagic anemia in dogs. Laurens and Mayerson (31), using carbon and mercury arc irradiation with dogs suffering from hemorrhagic anemia, found a rapid rise in reticulocytes and red blood cells, with little or no response in hemoglobin. Using artificial sunlight from the General Electric Sunlight Mazda lamp, we have found, in a preliminary study, somewhat the same effects in anemic young rats on whole milk diets.

Claims have also been made for the amino acids in this connec-

tion.   Thus Fontes and Thivolle (32) and Drabkin and Miller (33) have observed a definite beneficial effect when certain amino acids are given to anemic animals.

This again brings up the much discussed question as to whether the deficiency in some types of secondary anemia might not after all be organic as well as inorganic in nature.   We believe, however, that these substances or agents are not supplying an organic deficiency, unless it can be proved that a protein deficiency exists, and this is certainly not the case where young rats are fed on milk, the proteins of which are known to be of high biological value.   On the other hand there is experimental evidence to indicate that the action of such factors as mentioned above might be due to a general stimulation of metabolism as a whole (see Papers V and VI of this series).   Increased growth of the body and maturation of red blood cells from reticulocytes are always obtained when optimum doses of other elements are fed with Fe.   Irradiation in various ways gives a rapid rise in erythrocytes.   It is possible that the action of the amino acids may be due to their well known specific dynamic action.

The results given in Table VII demonstrate that the dosage of the element used as a supplement to the Fe in this type of experiment greatly influences the results that one may expect to obtain. It is possible that the failure of Hart, Steenbock, and coworkers (34) and Lewis and associates (35) to secure stimulation when some of the above elements were fed with Fe may have been due to the fact that their dosages were unsuited to give recovery.   For example, Hart, Steenbock, and coworkers (34) fed Mn in daily doses of 5.0, 0.25, and 0.01 mg. with Fe, and obtained negative results.   Underhill, Orten, and Lewis (9), feeding 5.0 and 1.0 mg. of Mn, likewise failed to secure recovery.   In our hands all doses of this element, with the exception of 0.1 mg., also failed to give any quicker recovery than 6 weeks.   Titus, Cave, and Hughes (6) and Goerner (7) found that 0.1 mg. of Mn gave recovery with Fe.

It would seem that optimum daily doses of each effective element used in this study as a supplement to Fe (these seem to fall within a narrow range) stimulate blood regeneration, while smaller doses have little or no effect, and larger ones may be toxic and cause anemia.   In certain cases polycythemia may be produced

The question as to whether Fe must be supplemented with Cu or some other element to bring about recovery in the nutritional anemia of the rat, and the corollary as to whether Fe should always be supplemented with some other element in the treatment of anemia we do not believe can be definitely answered at the present time. Hart, Steenbock, Waddell, and Elvehjem (5), Krauss (8), Lewis, Weichselbaum, and McGhee (35), and Underhill, Orten, and Lewis (9) have secured hemoglobin regeneration in rat anemia only when their Fe was supplemented with Cu, while Mitchell and Schmidt (36), Drabkin and Waggoner (37), Keil and Nelson (38), and the present authors have obtained recovery with Fe alone. Although Titus, Cave, and Hughes (6) were unable to obtain recovery with Fe alone, they found that it was effectively supplemented by Mn as well as Cu. Whipple and Robscheit-Robbins (39) in their hemorrhagic anemia of dogs apparently do not regard other elements as essential to Fe, while Hart, Elvehjem, and Steenbock (13) have been able to secure recovery in, and prevention of nutritional anemia of, the pig with pure Fe alone. There seems to be little doubt of the supplementing action of all the elements we have studied with 0.5 mg. of Fe, except Mg, Co, and Al, but our experiments do not indicate that it is necessary that Fe be supplemented with some other element or elements to secure blood regeneration.

The experiments here reported are the first, we believe, to show that the following elements: Ni, Ti, Zn, Rb, V, Cr, Se, and Hg, exert a supplementing action to Fe in the nutritional anemia of the rat.

After having noted the erythropoietic action on the part of many of the elements studied in this paper, it was recalled that Müller was attracted to the study of Ge in this connection on account of the fact that it is adjacent to arsenic in the periodic system. Although we were aware that the atomic numbers of these elements stood close to each other, we were somewhat surprised at the findings of the present studies. All of the elements reputed to be of therapeutic value in anemia are close to Fe and As in the series, except Hg, as shown in Table VIII. Although this is an attractive hypothesis, there does not seem to be sufficient reason for attaching much importance to the position of the various elements

TABLE VIII

Atomic Numbers and Elements Reputed to Have Erythropoietic Action

Atomic No.	Element	Elements recommended therapeutically (so far as known)	Erythropoietic action observed in nutritional anemia of rat (when added to 0 5 mg. Fe daily)
22	Titanium		Marked, 0.1 mg.
23	Vanadium	Yes	" 0.05 "
24	Chromium		" 0.05 "
25	Manganese	Yes	" 0.10 "
26	Iron	"	" 0.25 to 2 mg.
27	Cobalt		Slight
28	Nickel		Marked, 0.05 mg.
29	Copper	Yes	" 0.05 "
30	Zinc	"	Good, 0.05 to 0.1 mg.
31	Gallium*		(Not tested)
32	Germanium	Yes	Marked, 0.05 to 0.5 mg. -
33	Arsenic	"	" 0.01 mg.
34	Selenium		" 0.05 "
37	Rubidium		" 0.05 "
80	Mercury	Yes	" 0.04 to 0.15 mg.

* Schwartz and Sieke (40) found that gallium in rather large doses was toxic and produced anemia.

in the periodic system. Their Fe-supplementing action is probably dependent upon a catalytic effect.

SUMMARY AND CONCLUSIONS

184 young rats, rendered anemic by feeding on whole milk for 5 to 8 weeks after weaning, were studied in regard to the erythropoietic action of sixteen inorganic elements, when each was fed daily with 0.5 mg. of iron. The results obtained seem to justify the following conclusions.

1. Copper, nickel, germanium, manganese, arsenic, titanium, zinc, rubidium, chromium, vanadium, selenium, and mercury, each, fed separately in the proper doses with 0.5 mg. of Fe, brought about hemoglobin regeneration in 2 to 3 weeks and erythrocyte recovery in 2 to 2.5 weeks, as compared with 6 and 3.8 weeks, respectively, on this amount of Fe alone. Co, Mg, and Al were essentially ineffective in this connection.

2. The minimum effective daily dose (taking hemoglobin recovery on 0.5 mg. of Fe alone as 6 weeks) of these elements with

0.5 mg. of Fe was as follows: Cu, 0.025 mg.; Ni, 0.05 mg.; Ge, probably 0.05 mg.; As, 0.01 mg.; Mn, 0.1 mg.; Ti, probably 0.1 mg.; Zn, 0.1 mg.; Rb, V, Cr, and Se, probably 0.05 mg.; Hg, 0.04 mg. These are considered the optimum doses of these elements with Fe in nutritional anemia of the rat.

3. Hemoglobin and red blood cell regeneration in nutritional anemia can be either prolonged, hastened, or obtained in 6 weeks, depending upon the dosage of the element used as the supplement to the 0.5 mg. of Fe.

4. Optimum daily doses of all effective elements have also been fed to anemic young rats with daily doses of Fe from 0.05 to 0.25 mg. Cu was the most effective element for hemoglobin regeneration with 0.05 and 0.10 mg. of Fe, followed closely by Mn, As, and Cr. V was the most effective element with 0.15 and 0.20 mg. of Fe, followed closely by Cu, Mn, and Cr. Cr was the most effective element with 0.25 mg. of Fe, followed closely by Rb, Ge, and V.

5. Iron is essential for hemoglobin regeneration in nutritional anemia, and the action of these other elements is probably catalytic in nature.

6. The specificity of copper as a supplement to iron in the cure of the nutritional anemia of the rat receives little support from these studies.

BIBLIOGRAPHY

1. Wolf, W., Z. physiol. Chem., 26, 442 (1898).
2. Loewi, O., in von Noorden, C., Handbuch der Pathologie des Stoffwechsels, Berlin, 2nd edition, 2, 755–767 (1907).
3. Hare, H. A., A text book of practical therapeutics, Philadelphia and New York, 11th edition (1905).
4. Hammett, F. S., Nowrey, J. E., and Müller, J. H., J. Exp. Med., 35, 173 (1922).
5. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., J. Biol. Chem., 77, 797 (1928).
6. Titus, R. W., Cave, H. W., and Hughes, J. S., J. Biol. Chem., 80, 565 (1928).
7. Goerner, A., J. Lab. and Clin. Med., 15, 119 (1929).
8. Krauss, W. E., J. Biol. Chem., 90, 267 (1931).
9. Underhill, F. A., Orten, J. M., and Lewis, R. C., J. Biol. Chem., 91, 13 (1931).
10. Mitchell, H. S., and Miller, L., J. Biol. Chem., 85, 355 (1929–30).

11. Elden, C. A., Sperry, W. M., Robscheit-Robbins, F. S., and Whipple, G. H., *J. Biol. Chem.*, **79**, 577 (1928).
12. Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **84**, 131 (1929).
13. Hart, E. B., Elvehjem, C. A., and Steenbock, H., *J. Nutr.*, **2**, 277 (1930).
14. Beard, H. H., and Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, **26**, 510 (1929).
15. Myers, V. C., and Beard, H. H., *J. Am. Med. Assn.*, **93**, 1210 (1929).
16. Beard, H. H., and Myers, V. C., *J. Biol. Chem.*, **87**, p. xxxix (1930).
17. Nowrey, J. H., *Bull. Johns Hopkins Hosp.*, **35**, 180 (1924).
18. Mitchell, H. S., and Miller, L., *J. Biol. Chem.*, **92**, 421 (1931).
19. Keyes, E. L., *Am. J. Med. Sc.*, **71**, 17 (1876).
20. Tiffeneau, M., Dictionnaire de physiologie, Paris, **10**, 761 (1924).
21. Hammett, F. S., and Nowrey, J. E., *J. Exp. Med.*, **35**, 507 (1922).
22. Müller, J. H., and Iszard, M. S., *Am. J. Med. Sc.*, **163**, 364 (1922).
23. Lenker, J. L., *Penn. Med. J.*, **26**, 86 (1922).
24. Kast, L., Croll, H. M., and Schmitz, H. W., *J. Lab. and Clin. Med.*, **7**, 643 (1922).
25. Schmitz, H. W., *J. Lab. and Clin. Med.*, **9**, 374 (1924).
26. Bailey, G. H., Davidson, P. B., and Bunting, C. H., *J. Am. Med. Assn.*, **84**, 1722 (1925).
27. Minot, G. R., and Sampson, J. J., *Boston Med. and Surg. J.*, **189**, 629 (1923).
28. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, **72**, 419 (1925).
29. Furniss, A., *Am. J. Physic. Therap.*, **7**, 465 (1931).
30. Osato, S., and Tanaka, S., *Z. ges. exp. Med.*, **65**, 692 (1929).
31. Laurens, H., and Mayerson, H. S., *J. Nutr.*, **3**, 465 (1931).
32. Fontes, G., and Thivolle, L., *Compt. rend. Soc. biol.*, **105**, 965 (1930).
33. Drabkin, D. L., and Miller, H. K., *J. Biol. Chem.*, **90**, 531 (1931).
34. Waddell, J., Steenbock, H., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **84**, 115 (1929).
35. Lewis, G. T., Weichselbaum, T. E., and McGhee, J. L., *Proc. Soc. Exp. Biol. and Med.*, **27**, 329 (1930).
36. Mitchell, H. S., and Schmidt, L., *J. Biol. Chem.*, **70**, 471 (1926).
37. Drabkin, D. L., and Waggoner, C. S., *J. Biol. Chem.*, **89**, 51 (1930).
38. Keil, H. L., and Nelson, V. E., *Proc. Soc. Exp. Biol. and Med.*, **28**, 392 (1931).
39. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, **92**, 362 (1930).
40. Schwartz, L., and Sieke, F., *Arch. Hyg.*, **100**, 143 (1928).

STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

III. THE PREVENTION OF ANEMIA BY MEANS OF INORGANIC ELEMENTS

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In the previous papers of this series (1, 2) experimental evidence
is furnished to show that Fe alone or Fe supplemented by many
other inorganic elements has a very definite beneficial effect upon
blood regeneration in the nutritional anemia of the rat. Since
this evidence appears so convincing it would hardly seem neces-
sary to make a study of the prophylactic effect of these elements
in this type of anemia. It is only logical to believe that if an ele-
ment can bring about regeneration of hemoglobin and erythrocytes
in anemic young rats, it will also prevent the onset of anemia in
these animals under similar experimental conditions.

As a result of a recent study, however, Krauss (3, 4) concludes
that Fe alone is valueless in preventing nutritional anemia in the
rat, unless it is supplemented with Cu. Titus and Hughes (5) were
likewise unable to prevent the development of anemia on a milk
diet supplemented with Fe.

The results of the present study indicate that both inorganic
Fe and Fe supplemented with several other metals have a definite
action in preventing nutritional anemia of young rats. It should
be noted, however, that none of these elements with milk was quite
as good as the stock diet in this respect.

The technique used was the same as described in Paper I of this
series. The supplements were fed daily with whole milk to young
rats for a period of about 6 to 8 weeks after weaning. The data
are given in Tables I and II.

DISCUSSION

The results of this study demonstrate that Fe (as the H_2S filtrate of $FeCl_3$ prepared from electrolytic Fe) when added to the

TABLE I

Effect of Inorganic Iron in Prevention of Nutritional Anemia

Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Duration of experiment		Diet
	Before	After	Before	After	Before	After	Hb	R.b.c.	
	gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*	
499	35	204	7.5	16.6	4.7	9.1	6	6	Stock diet
500	34	125	7.2	16.1	8.1	10.5	6	6	
501	33	175	7.7	12.5	4.5	7.5	6	6	
502	31	156	7.1	11.1	4.7	7.3	6	6	
503	35	151	9.4	17.3	7.8	8.8	6	6	
504	35	154	10.7	17.0	9.1	11.0	6	6	
505	35	133	11.7	17.0	6.3	8.1	6	6	
Average..	34	157	8.8	15.4	6.5	8.9	6.0	6.0	
506	35	159	11.7	5.0	6.5	5.4	9	9	Milk alone
507	25	113	8.9	3.0	6.0	3.0	7	7	
508	31	151	9.0	3.5	5.5	2.6	7	7	
509	34	102	9.4	3.0	7.9	3.9	6	6	
510	26	91	8.8	4.0	6.2	4.1	6	6	
511	32	120	8.8	3.5	6.7	3.3	6	6	
Average..	31	123	9.4	3.7	6.5	3.7	6.8	6.8	
512	20	166	7.2	13.2	6.3	7.1	9	3	Milk + 0.25 mg. Fe
513	28	132	8.4	11.8	6.3	8.0	9	3	
514	28	129	6.7	13.0	5.4	8.7	6	4	
515	24	159	9.8	13.0	6.2	7.3	6	4	
516	26	119	9.1	10.0	4.9	7.7	6	6	
517	21	94	9.1	10.5	6.4	7.1	6	6	
518	24	94	7.7	10.5	5.9	11.2	6	6	
519	28	103	7.7	10.0	4.9	9.3	6	6	
520	22	85	7.5	10.0	4.1	7.5	6	6	
521	24	84	6.7	10.0	5.2	7.0	6	6	
522	25	65	6.7	11.8	3.9	8.1	6	6	
523	32	95	6.6	11.1	3.7	7.7	6	6	
524	30	75	7.2	11.1	4.0	8.6	6	6	
525	35	95	6.8	11.5	3.4	7.6	6	6	
	26	106	7.7	11.2	5.0	8.1	6.4	5.3	

TABLE I—*Concluded*

Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Duration of experiment		Diet
	Before	After	Before	After	Before	After	Hb	R.b.c.	
	gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	*wks.*	*wks.*	
526	25	158	9.5	17.3	6.2	9.3	4	3	Milk + 0.5
527	23	105	10.5	8.4	6.4	7.4	5	3	mg. Fe
528	26	73	9.2	11.8	6.4	8.6	5	3	
529	22	81	9.9	10.8	6.6	7.5	6	3	
530	43	146	12.0	12.8	7.8	9.8	6	6	
531	34	110	13.0	14.3	10.0	9.3	6	6	
532	31	105	12.1	13.8	9.6	10.4	6	6	
Average..	29	111	10.9	12.8	7.6	8.9	5.4	4.3	

milk we have employed is effective in preventing nutritional anemia in young rats. Hemoglobin maintenance was not quite up to normal with 0.25 mg. of Fe daily, but with 0.5 mg. it reached about 13 gm. per 100 cc., a value we have considered normal in the present studies. Fe supplemented with Cu, Ni, Mn, or Zn, did not give any higher average maintenance of hemoglobin than Fe alone. Animals on the stock diet had an average value for hemoglobin of 15.4 gm. per 100 cc. after a 6 weeks period. Since our milk apparently contains all the nutritive factors, except Fe, for growth from weaning until 100 gm. of body weight is attained, it would seem that the difference between the controls and experimental animals might have been due to the fact that the controls were able to consume more food than the experimental animals on milk. Erythrocyte maintenance was excellent on all diets.

These results do not confirm the findings of Krauss (3) and Titus and Hughes (5) that Fe fails to prevent the development of nutritional anemia in young rats on whole milk diets. In later work Krauss (4) attempted to discover the component or components of synthetic rations which would adequately supplement a milk diet, and found that yeast, casein, starch, agar, and McCollum's Salt Mixture 185 were effective in preventing nutritional anemia. He ascribed this to the amounts of Fe and Cu furnished the animals by each substance. It should be noted that he used only one animal of a group of four for the weekly hemoglobin

determination. In a later study on the effect of different yeasts on the prevention and cure of nutritional anemia, Krauss (6) con-

TABLE II

Effect of Inorganic Elements with Fe in Prevention of Nutritional Anemia

Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Duration of experiment		Diet
	Before	After	Before	After	Before	After	Hb	R.b.c.	
	gm.	gm.	gm.	gm.	mil-lions	mil-lions	wks.	wks.	
533	32	95	6.8	11.1	3.7	7.7	6	6	0.25 mg. Fe
534	30	75	7.0	11.1	4.0	8.5	6	6	+ 0.05 mg.
535	35	90	8.2	11.5	3.4	7.6	6	6	Cu
536	38	105	9.1	12.5	4.1	8.5	6	6	
537	32	124	10.1	14.3	4.1	8.6	6	6	
538	28	120	9.6	12.5	3.9	7.7	6	6	
Average..	33	102	8.5	12.2	3.9	8.1	6	6	
539	62	116	6.8	11.1	4.0	6.7	6	6	0.25 mg. Fe
540	36	94	6.5	11.1	4.6	8.1	6	6	+ 0.05 mg.
541	63	122	7.2	10.0	5.1	8.3	6	6	Ni
Average..	54	111	6.8	10.7	4.6	7.7	6	6	
542	61	101	6.8	10.3	6.8	8.1	6	6	0.25 mg. Fe
543	45	98	7.2	11.1	5.6	7.9	6	6	+ 0.1 mg.
544	34	73	9.3	10.2	4.9	7.8	6	6	Mn
545	36	79	8.6	10.5	5.3	8.1	6	6	
546	36	141	9.2	10.8	7.7	9.6	7	7	
547	29	111	7.0	10.8	5.8	9.8	7	7	
Average..	40	101	8.0	10.6	6.0	8.6	6.3	6.3	
548	81	130	6.8	10.5	4.7	7.2	6	6	0.25 mg. Fe
549	35	96	7.2	11.1	3.9	7.5	6	6	+ 0.01 mg.
550	66	116	9.0	11.1	5.4	6.9	6	6	As
551	33	142	13.3	12.1	10.2	10.0	7	7	
552	36	113	9.9	13.0	9.6	9.6	7	7	
553	34	110	13.0	12.1	8.0	9.2	7	7	
Average..	48	118	9.9	11.7	7.0	8.4	6.5	6.5	

cluded that the antianemic effect of these yeasts was not due to Fe but to Cu. In view of the fact that yeast contains several sub-

stances of high nutritive value, it does not seem to us that Krauss has definite proof for his conclusion that the Cu content of the yeast was the chief factor in preventing the anemia.

It was observed in this laboratory that Fe solutions supplied to us by Dr. W. E. Krauss of the Ohio Agricultural Experiment Station and Dr. C. A. Elvehjem of the Wisconsin laboratory prevented the onset of anemia in a few of our normal young rats on a whole milk diet. For results see Tables III and IV of Paper I of this series.

SUMMARY AND CONCLUSIONS

As a result of this and previous studies on the effects of inorganic elements in the prevention and cure of nutritional anemia in young rats fed on whole milk diets, the following conclusions seem justified.

1. Inorganic Fe (as the H_2S filtrate of $FeCl_3$ prepared from electrolytic Fe) appears to have both a prophylactic and curative action in this type of anemia.

2. Fe, supplemented by Cu, Ni, Mn, or As, does not seem to be appreciably better in preventing anemia than Fe alone.

BIBLIOGRAPHY

1. Beard, H. H., and Myers, V. C., *J. Biol. Chem.*, **94**, 71 (1931).
2. Myers, V. C., and Beard, H. H., *J. Biol. Chem.*, **94**, 89 (1931).
3. Krauss, W. E., *J. Dairy Sc.*, **12**, 242 (1929).
4. Krauss, W. E., *J. Dairy Sc.*, **12**, 438 (1929).
5. Titus, R. W., and Hughes, J. S., *J. Biol. Chem.*, **83**, 463 (1929).
6. Krauss, W. E., *J. Dairy Sc.*, **13**, 246 (1930).

STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

IV. THE PRODUCTION OF HEMOGLOBINEMIA AND POLYCYTHEMIA IN NORMAL ANIMALS BY MEANS OF INORGANIC ELEMENTS

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There are a considerable number of observations in the literature which show that several inorganic elements, when administered in suitable doses, will produce polycythemia. Among these may be mentioned arsenic, manganese, phosphorus, germanium, cobalt, and copper. Conversely, chronic poisoning with certain elements may lead to anemia. Lead is a notable example. Poisoning with arsenic, vanadium, and gallium has also been shown to produce anemia.

Polycythemia has been noted in phosphorus poisoning by Silbermann (1), while Steiger (2) observed an increase of about a million red blood cells after the administration of As and P. Polycythemia was also noted by Schreyer (3) after the administration of both Mn and Fe. He suggests that the polycythemia may be due to increased cell respiration as the result of the presence of abnormal amounts of Fe or Mn as an oxidation catalyzer. A number of investigators have shown that GeO_2 is capable of producing polycythemia, following the pioneer observations of Hammett, Nowrey, and Müller (4) on the rat. In a later paper Hammett and Nowrey (5) made a histological study and found evidence of a marked stimulation in the formation of nucleated erythrocytes in the bone marrow of their rats given germanium. Large doses of a number of metals in the form of their salts have been fed by Waltner (6), the salts being pulverized and incorporated in McCollum's stock diet. With Co in a concentration of 0.5 and 2

per cent, there was a striking increase in erythrocytes and hemoglobin within 9 days.   When 0.5 per cent Co was given there was only slight loss of body weight, length of life was 6 months, and the hemoglobin increase amounted to 160 to 170 per cent and erythrocytes to 10 to 11 millions per c. mm.   The Co polycythemia and polychromemia were present until the time of death.   In his studies on the toxicity of Cu, De Moor (7) calls attention to the production of hemoglobinemia.

In the preceding papers of this series it was shown that a number of inorganic elements produce rapid blood regeneration in the nutritional anemia of the rat when fed daily with 0.5 mg. of Fe. In a few experiments values for red blood cells and hemoglobin much above normal were observed after 2 to 3 weeks upon the supplements.   This being the case it seemed desirable to ascertain if the continued action of these elements would produce further stimulation of the hematopoietic organs.

<div align="center">EXPERIMENTAL</div>

The early anemia experiments were usually terminated when 13 to 14 gm. of hemoglobin per 100 cc. and 7 to 8 millions of erythrocytes per c. mm. were obtained.   In later studies the experimental animals were allowed to continue upon their supplements (Fe, Fe and Cu, Ge, Mn, Ni, and As) after regeneration of the blood was complete for periods of 3 to 5 weeks.   Other animals, after complete recovery, were placed on Sherman's Diet B[1] for several weeks, after which determinations of hemoglobin and red blood cells were again made.   The rats were kept in separate cages and fed on the above diet, supplemented with either 0.5 or 1 per cent of Co, V, Fe, Cu, Mg, Mn, Zn, and Ni, in the form of the solid sulfate or chloride.

Rats on Milk Diet with Small Doses of Mineral Supplements

When 0.1 mg. of Ge, Zn, or Mn was fed daily with Fe in milk after recovery from anemia, increases above the normal in both red blood cells and hemoglobin were obtained (see Table I). With 0.05 mg. of Ni, 0.01 mg. of As, or 0.05 mg. of Cu plus 0.5 mg. of Fe, or the same amount of Fe alone, each fed daily in milk,

[1] The composition of the diets used is given in detail in Paper I.

there was no effect upon the erythrocytes, but hemoglobin values of 16 to 20 gm. per 100 cc. were usually found. It should perhaps be noted that two of the rats on 0.5 mg. of Fe alone failed to show any increase in hemoglobin. In practically every case the hemoglobin and red blood cells returned to normal in about 2 weeks after the high values were reached. Thus it would seem that these

TABLE I

Production of Hemoglobinemia and Polycythemia in Adult Animals by Means of Inorganic Elements

Average values.

No. of animals	Hb per 100 cc.			R.b.c. per c.mm.			Diet
	Before	After	Increase	Before	After	Increase	
	gm.	*gm.*	*per cent*	*millions*	*millions*	*per cent*	
4	12.8	20.0	56.2	8.4	**12.8**	53.4	1 per cent Co in stock diet
3	14.2	19.4	36.5	7.7	10.6	37.6	Whole milk + 0.5 mg. Fe + 0.1 mg. Zn daily
2	13.3	19.4	45.8	8.4	10.3	22.6	0.5 per cent V in stock diet
2	16.0	18.0	12.5	7.9	**13.3**	68.3	1 per cent V in stock diet
6	16.0	18.4	15.0	9.6	9.14	None	Whole milk + 0.5 mg. Fe + 0.05 mg. Cu daily
9	14.1	18.0	28.0	8.6	8.30	"	Whole milk + 0.5 mg. Fe daily
7	14.9	18.0	20.8	8.7	**12.6**	44.8	" " + 0.5 " " + 0.1 mg. Ge daily
3	13.0	18.0	38.4	8.0	9.8	22.5	Whole milk + 0.5 mg. Fe + 0.1 mg. Mn daily
3	13.6	17.1	25.8	8.3	8.7	4.8	Whole milk + 0.5 mg. Fe + 0.05 mg. Ni daily
4	13.6	16.3	19.8	8.2	8.2	None	Whole milk + 0.5 mg. Fe + 0.01 mg. As daily

The bold-faced figures indicate particularly significant values.

elements have a powerful stimulating effect on hemoglobin formation, and in some cases on the erythrocytes, and that the stimulus is still present for a few weeks after recovery.

Rats on Stock Diet Plus 0.5 or 1 Per Cent of Mineral Supplements

With Co and V at a concentration of 1 per cent in the stock diet very high values for both red blood cells and hemoglobin were

TABLE II

Effect of Addition of 1 Per Cent Inorganic Elements to the Stock Diet upon the Concentration of Hemoglobin and Erythrocytes of Normal Animals

Rat No.	Body weight		Hb per 100 cc.		R.b.c. per c.mm.		Element added to stock diet
	Before	After	Before	After	Before	After	
	gm.	*gm.*	*gm.*	*gm.*	*millions*	*millions*	
317	140	147	13.3	13.3	8.9	8.2	$FeSO_4$
318	142	158	13.3	10.5	8.6	6.7	
320	136	117	14.3	13.5	9.0	6.6	
321	125	138	13.3	10.5	9.0	6.5	$CuSO_4$
323*	124	152	13.8	9.1	8.2	7.7	
324*	151	170	14.3	12.5	9.8	8.9	
325	119	137	14.3	9.5	9.2	6.2	$MgCl_2 \cdot 6H_2O$
326	156	148	14.3	12.5	9.4	7.1	
328	178	191	14.3	12.5	7.2	7.4	
329	174	166	14.6	13.5	8.1	8.1	$MnCl_2$
330	143	167	16.6	12.5	7.2	7.5	
331	167	155	16.0	12.5	7.3	7.5	
332	162	175	14.3	11.1	8.8	8.1	$ZnCl_2$
333	148	172	14.3	11.1	7.8	9.2	
334*	183	111	14.3	11.7	7.5	8.1	
335*	118	91	14.3	13.3	7.5	8.6	$NiCl_2 \cdot 6H_2O$
336*	113	84	14.0	13.3	7.1	9.0	
337*	156	118	16.6	9.1	8.4	7.6	
341	161	158	13.3	12.5	7.8	8.2	Cr_2Cl_6
342	147	162	13.3	13.3	7.5	8.1	
343	169	146	13.3	13.3	7.8	8.7	
344	125	155	13.3	14.3	8.2	8.3	Se powder
345	127	146	13.2	13.3	8.0	7.5	
346	225	240	13.1	10.0	10.7	7.5	$AlCl_3$
347	268	184	14.8	9.1	10.3	9.7	
348	293	269	13.8	12.5	8.4	8.6	
349	273	259	15.5	14.3	8.6	8.2	

* Animal died.

obtained. For example Rat 185 gave a 94 per cent increase in cells and a 71 per cent increase in hemoglobin. In this concentration these elements were toxic and in the case of Co an intense hyperemia was present at death. One rat from both the Co and V experiments survived, and after removal of the metals from the diets of these two animals the hemoglobinemia and polycythemia were still present for several weeks.

The results obtained when 1 per cent of the following elements, Fe, Cu, Mg, Mn, Zn, Ni, Cr, Se, and Al, were fed in the stock diet are given in Table II. Cr, Se, and Ni had no effect upon the hemoglobin or cells. (One animal on Ni did show a drop to 7.5 gm. in hemoglobin.) With Fe both values decreased somewhat, while with Cu, Mg, Zn, and Al there was no change in the cells, but a definite drop in hemoglobin. A marked loss of weight occurred in the animals on Ni, and this element and Cu appeared to be the most toxic.

DISCUSSION

It will be seen then that the present experiments confirm the production of polycythemia by a number of inorganic supplements reported in the literature and in addition demonstrate a similar action for V.

The question may well be raised as to whether the polycythemia and hemoglobinemia found may not have been due to changes in blood volume. We have no data directly answering this point, but do not believe that this is the explanation. This and similar questions have been carefully discussed by Lamson (8). It is of interest to note in this connection that a lack of inorganic elements in the diet may cause polycythemia, as shown by Smith and Swanson (9). When 130 gm. rats were given a diet containing only 0.5 per cent ash, they found a true polycythemia, small red blood cells with a diminished concentration of hemoglobin.

SUMMARY AND CONCLUSIONS

Studies have been conducted upon anemic young rats which continued to receive mineral supplements with their milk for a few weeks after complete blood regeneration, and upon adult rats which were given the stock diet plus 0.5 or 1 per cent of several inorganic supplements, with the following results.

0.5 per cent V in the stock diet produced a large increase in hemoglobin and a considerable increase in red blood cells. 1 per cent of both V and Co produced a marked hemoglobinemia and polycythemia.

0.1 mg. of Ge fed daily in milk with 0.5 mg. of Fe produced a marked polycythemia and a moderate hemoglobinemia.

0.1 mg. of Zn, 0.1 mg. of Mn, 0.05 mg. of Cu, 0.05 mg. of Ni, and 0.01 mg. of As plus 0.5 mg. of Fe in milk resulted in the production of a moderate hemoglobinemia (increase in hemoglobin of 16.3 to 19.4 gm. per 100 cc.). With 0.5 mg. of Fe alone the increase in hemoglobin was nearly as great (to 18.0 gm.) as with the additional inorganic supplements.

The polycythemia and hemoglobinemia caused by several inorganic elements furnish additional proof of the stimulating action of a number of elements in hemoglobin and red blood cell metabolism.

BIBLIOGRAPHY

1. Silbermann, R., *Prag. med. Woch.*, **32**, 167 (1907).
2. Steiger, O., *Med. Klin.*, **2**, 1746 (1912).
3. Schreyer, W., *Deutsch. Arch. klin. Med.*, **148**, 149 (1925).
4. Hammett, F. S., Nowrey, J. E., Jr., and Müller, J. H., *J. Exp. Med.*, **35**, 173 (1922).
5. Hammett, F. S., and Nowrey, J. E., Jr., *J. Exp. Med.*, **35**, 507 (1922).
6. Waltner, K., *Arch. exp. Path. u. Pharmakol.*, **141**, 123 (1929).
7. De Moor, L., *Arch. pharmacod.*, **1**, 81 (1894–95).
8. Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, **7**, 169 (1915).
9. Smith, A. H., and Swanson, P. P., *Am. J. Physiol.*, **90**, 517 (1929).

STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

V. THE ACTION OF IRON AND IRON SUPPLEMENTED WITH OTHER ELEMENTS UPON THE DAILY RETICULOCYTE, ERYTHROCYTE, AND HEMOGLOBIN RESPONSE

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An increase in the reticulocyte count in different anemic states is believed by clinicians to be indicative of blood regeneration. In fact, the effectiveness of liver or liver extract in pernicious anemia is usually judged by the reticulocyte response, and if a distinct improvement in health does not occur in 4 weeks, Minot (1) is of the opinion that the patient is not suffering from pernicious anemia.

In order to obtain more evidence of the effect of Fe alone, and Fe supplemented by other elements, we have made reticulocyte counts by the excellent method of Friedlander and Wiedemer (2), red blood cell counts as outlined in Paper I of this series, and hemoglobin estimations by the pseudoperoxidase method described by Bing and Baker (3). The above estimations were made daily for the 1st week and at stated intervals thereafter. All animals were kept in large glass museum jars, described in Paper I of this series, with a raised glass network for a base. The Fe solutions used in some of the experiments were supplied by Doctor C. A. Elvehjem of the University of Wisconsin and Doctor W. E. Krauss of the Ohio Agricultural Experiment Station. These investigators had reported negative results upon blood regeneration with Fe in nutritional anemia of the rat. In several other cases the H_2S filtrate of electrolytic Fe (used in about 80 per cent of the experiments reported in this series of papers) was used. Identical results were obtained with all Fe preparations. The remaining animals

received optimum doses of other elements together with 0.25 mg. of Fe daily.

The daily milk intake of all the experimental rats varied from 25 to 50 cc. during the period of these studies, which is an adequate intake of milk with Fe for blood regeneration during the first 2 weeks.   The data are given at the bottom of the charts.   The
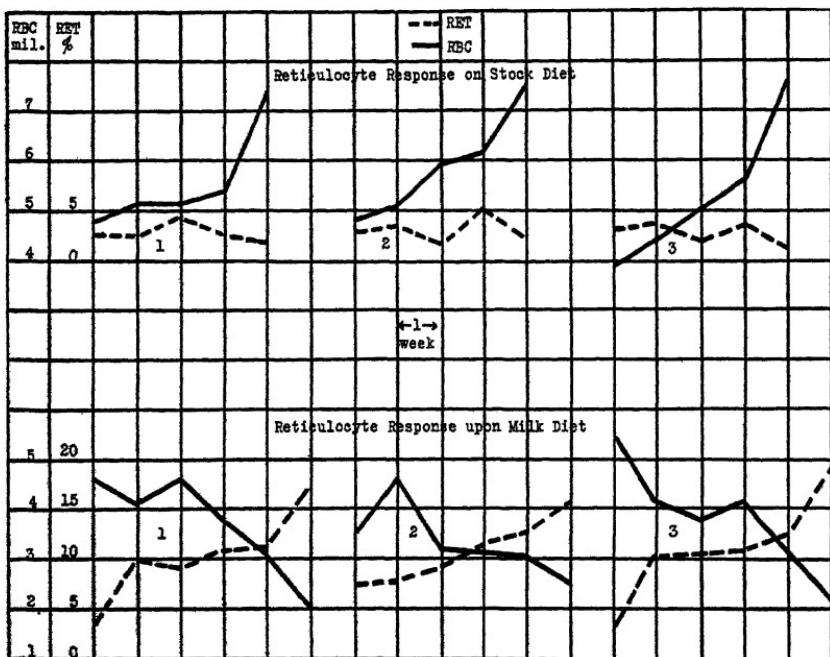
CHART I.   Reticulocyte response of young rats upon the stock diet and whole milk diet.

importance of an adequate milk intake in addition to mineral supplements in this type of study cannot be overemphasized.

Experimental Observations

The results obtained are shown graphically in the form of charts. Chart I gives the weekly erythrocyte counts and reticulocytes, as percentage of the erythrocytes, from the time of weaning until the normal cell count is obtained.   After 4 weeks feeding on the stock diet a count of 7 to 8 million erythrocytes was found, 1 to

5 per cent of which was present as reticulocytes. The average was about 3 per cent. These were normal young rats. At the bottom of the chart are shown three litter mates of the above, which developed anemia on whole milk diets. A progressive drop in cells and progressive increase in percentage of reticulocytes will be noted. When the anemic level at 6 weeks is reached there are about 2 to 3 million erythrocytes per c. mm. and 15 to 20 per
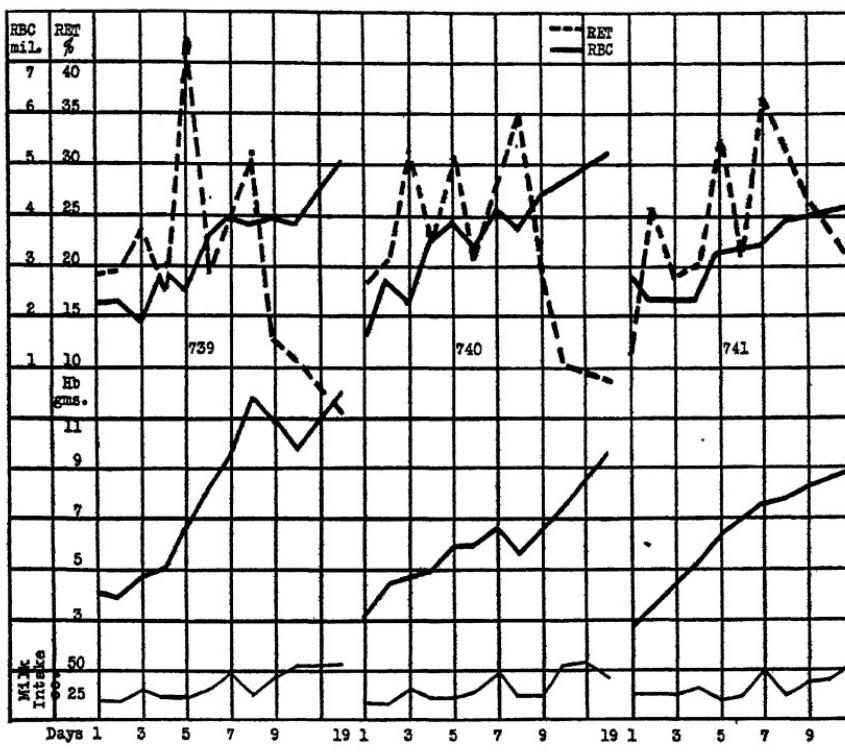
CHART II. Reticulocyte response with iron

cent reticulocytes. The same observations were obtained with all experimental rats in this study.

It will be observed that the nutritional anemia of the rat on whole milk diets is not accompanied by a deficiency of reticulocytes, but rather a lack of changing of reticulocytes into mature red blood cells. It is generally believed that the reticulocyte is a

form of immature red blood cell. Further evidence for these points of view will be presented below.

Chart II gives the results obtained with three representative rats out of a total of ten, receiving 0.25 mg. of Fe daily. There was a gradual increase in erythrocytes and hemoglobin. The reticulocyte peak was reached usually in 4 days, after which there may be one or two more peaks, followed by a progressive drop
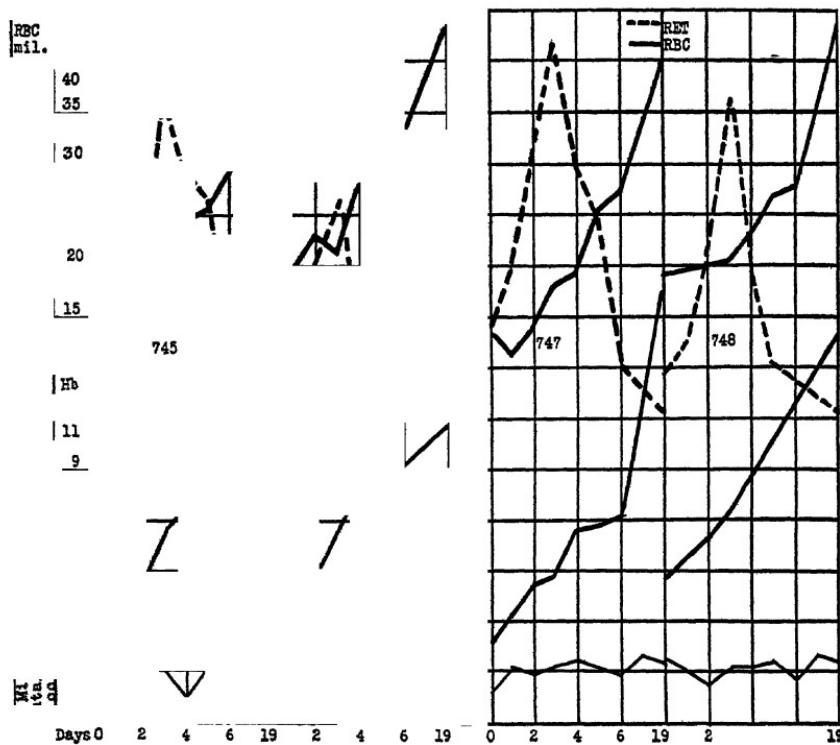
CHART III. Reticulocyte response with iron and copper

eventually to the normal level. The period covered by these peaks lasted for about 6 days.

Variable rates of individual hemoglobin increase for the 1st week or so have repeatedly been obtained in these studies. If no response occurs the 1st week, the conclusion cannot be drawn that the blood is not regenerating, since later on increases in hemoglobin

will occur.   On the other hand, very striking increases in hemoglobin may occur the 1st week (see Paper VI of this series).

Chart III with Fe and Cu shows that two rats reached a reticulocyte peak in 4 days as usual, with a big drop in reticulocytes the 5th day, while two others took 4 days for the drop in reticulocytes. The increases in red blood cells and hemoglobin were striking.

CHART IV. Reticulocyte response with iron and manganese

Chart IV gives the results obtained with Fe and Mn.   The same reticulocyte peak occurs at 4 days as with Fe alone, but the first big drop in reticulocytes always occurred on the 5th day, with a corresponding increase in red blood cells and hemoglobin soon thereafter.

Chart V gives the results for Fe and As.   The reticulocyte peak was reached as usual in 3 or 4 days, followed by a large de-

crease in reticulocytes with an increase in red blood cells the following day.

Chart VI gives the results with Fe and Zn, and Fe and Ge. Rat 753 on Fe and Zn began a transformation of reticulocytes into red blood cells the 1st day of treatment.   No reticulocyte peak occurred.   Similar results were obtained with Fe and Ge (Rat
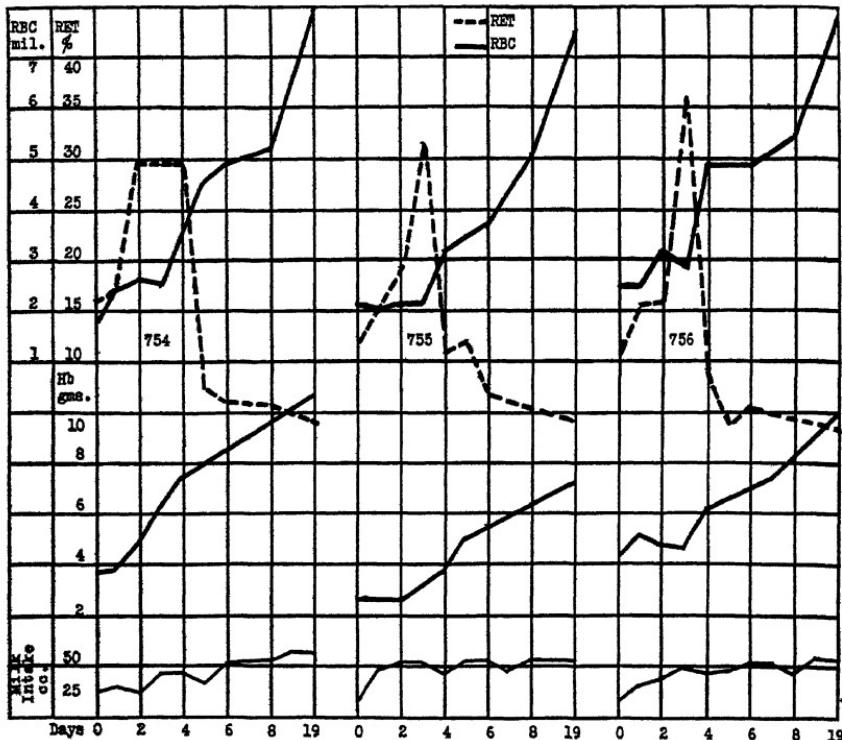
CHART V.   Reticulocyte response with iron and arsenic

757).   In this case there was an increase of 2.5 million red blood cells per c. mm. the first 2 days of treatment and an increase from 3 to 10 gm. of hemoglobin per 100 cc. the 1st week.

Chart VII shows the results for two more animals with Fe and Ge and two with Fe and V.   In the latter case it took 5 days for the reticulocytes to reach their highest peak, but the usual drop occurred the next day, with large increases in cells.   Rat 762 on

Fe and V showed an increase from 2 to 7.5 gm. of hemoglobin in 3 days.

DISCUSSION

According to Friedlander and Wiedemer (2) an increase in reticulocytes occurs in the following clinical conditions: hemorrhage, pregnancy, in the new born infant, lead poisoning, in persons work-
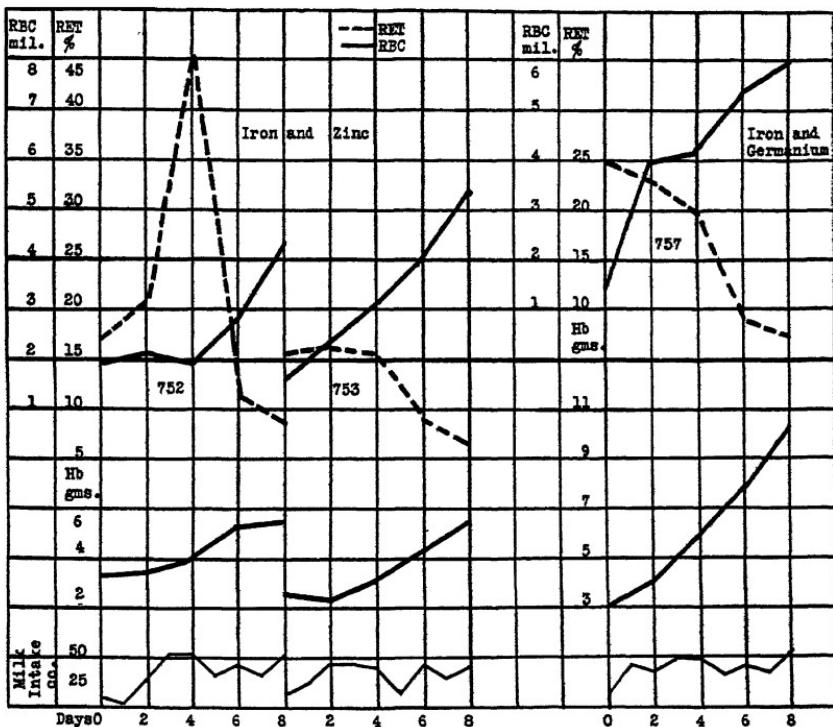
CHART VI.   Reticulocyte response with iron, zinc, and germanium

ing in lead plants, but not suffering from lead poisoning, hemolytic jaundice, etc.   It also appears to occur in the nutritional anemia of the rat.

The increase in reticulocytes observed in this study is no doubt similar to that which occurs in hemorrhagic anemia. In an attempt to make good the blood loss, the hematopoietic organs put increased numbers of these immature cells into the circulation.

In the nutritional anemia of the rat, these reticulocytes are not changed into mature red blood cells unless some form of adequate therapy is given.　If Fe is given there is a stimulation of reticulo-cytosis with a slow and gradual increase in red blood cells and hemoglobin.　Thus at least one of the functions of Fe in nutri-tional anemia is to aid in the production of new reticulocytes.
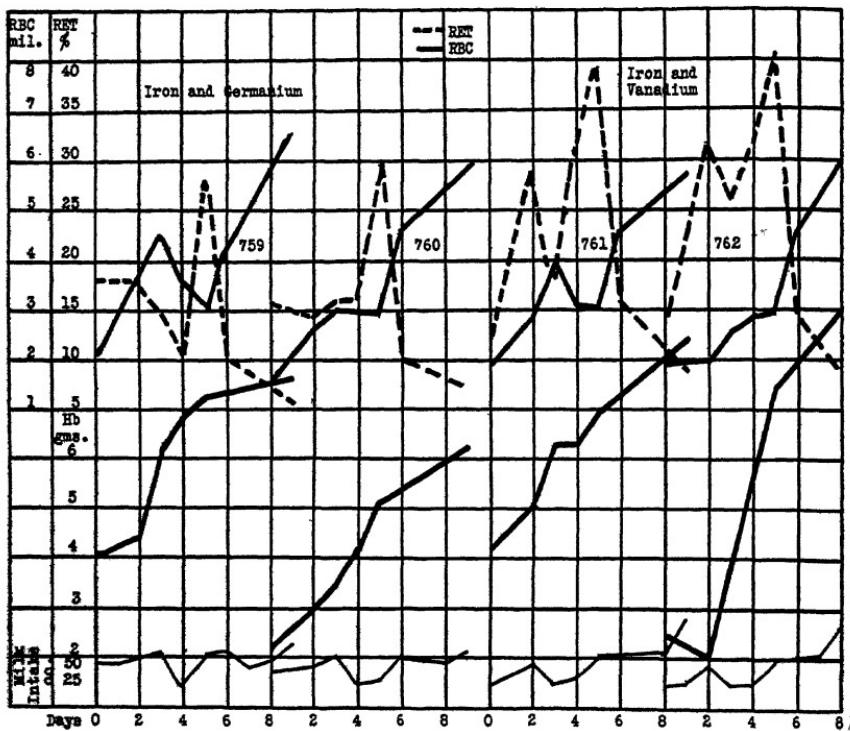
CHART VII.　Reticulocyte response with iron, germanium, and vanadium

This function of Fe has also been observed by Levi and Micheletti (4), Chatterjee (5), and Mettier and Minot (6).

When other elements were given with Fe there was always a rapid disappearance of reticulocytes and rapid increase in cells and hemoglobin in these anemic rats, and it would appear that the chief function of these other elements with Fe was to cause a quicker maturation of the red blood cells from reticulocytes and increase in hemoglobin than would occur with Fe alone.　Thus

DIAGRAM 1

Biochemical View of Pernicious and Nutritional Anemia

Pernicious anemia (man)	Nutritional anemia (rat)
Organic deficiency	Inorganic deficiency
(Dipeptide of hydroxyproline and hydroxyglutamic acid)	(Iron)
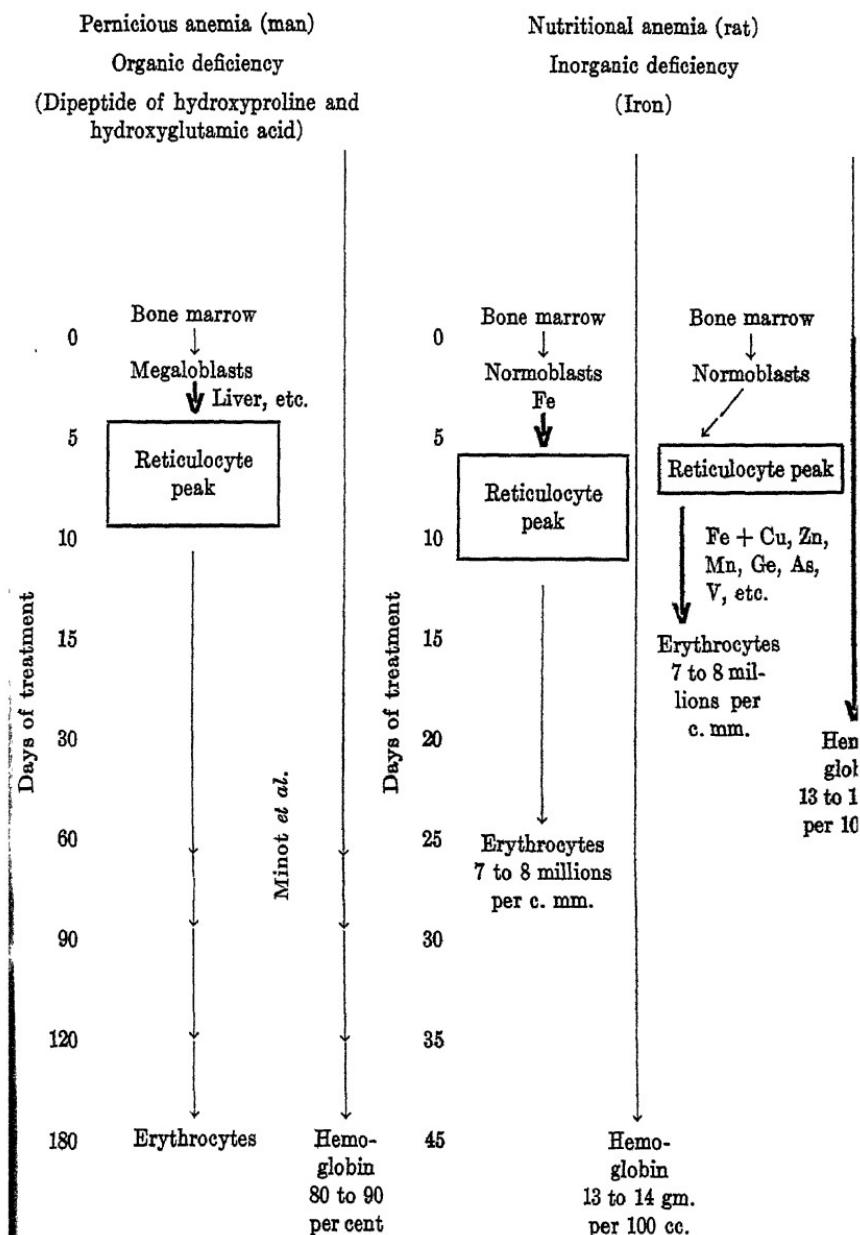
these elements would seem to catalyze the action of Fe.   This is shown by the fact that Fe starts the reticulocyte response (1 to 4 days), and thus enters into the morphological development of the red blood cells in the bone marrow, before the catalytic action of the other elements becomes manifest.   On the 5th day these elements show their effect in the big drop in reticulocytes and big increase in cells and hemoglobin.   Thus they speed up a morphological reaction already in progress, which is usually considered as a catalytic action.   These results also show that Fe is a true hematopoietic stimulant in that the organs in the red bone marrow produce new reticulocytes.   Whether maturation of these reticulocytes takes place in the bone marrow or in the blood stream is difficult to answer.   Faludi (7) stated that Mn, which gave quick improvement in seventeen cases of secondary anemia, stimulated the erythropoietic part of the bone marrow with a resulting formation of reticulocytes.

Another important effect of these elements with Fe is the increased growth always obtained when they are given in optimum doses (see Paper VI of this series).

A schematic comparison of pernicious and nutritional anemia is given in Diagram 1.   In pernicious anemia Whipple (8) believes the chief deficiency is a lack of stroma-building material.   It seems to be a lack of reticulocytosis, or a lack of transformation of the megaloblasts into reticulocytes.   The organic factor in liver (either the nitrogenous base or polypeptide of Cohn or the dipeptide of hydroxyglutamic acid and hydroxyproline of West and Dakin) will bring about a marked transformation of these megaloblasts into reticulocytes, which in due time are further transformed into mature red blood cells which then fill up with hemoglobin.   Fe does not seem to be of much benefit in pernicious anemia, nor does liver extract, owing to its lack of Fe, appear to afford much help in the nutritional anemia of the rat.   The nutritional anemia of the growing rat may be considered an inorganic Fe deficiency disease, since Fe will both prevent and cure the condition.   The action of Fe in nutritional anemia is analogous to that of liver or liver extract in pernicious anemia.   A complete bone marrow study of nutritional anemia, however, may be necessary fully to establish these points.

SUMMARY AND CONCLUSIONS

Daily reticulocyte counts in addition to erythrocyte counts and hemoglobin estimations were made in a group of normal young rats on the stock diet, a group of young rats developing anemia on whole milk diets, a group of rats receiving 0.25 mg. of Fe daily, and a group receiving optimum doses of other elements with 0.25 mg. of Fe. The results obtained show the following.

1. Rats at weaning (40 to 50 gm. body weight) have about 10 gm. of hemoglobin per 100 cc., with an average concentration of about 3 per cent reticulocytes. 4 weeks later the hemoglobin is 13 to 14 gm. per 100 cc., the red blood cell count, 7 to 8 millions per c. mm., with no change in the reticulocyte percentage.

2. In young rats developing anemia on a whole milk diet there occurs a progressive fall in red blood cells and hemoglobin with a progressive increase in the reticulocytes which are not transformed into mature red blood cells. Nutritional anemia in rats then appears to be due, among other things, to a lack of maturation of erythrocytes.

3. The administration of 0.25 mg. of Fe daily gave a reticulocyte peak, from 15 to 45 per cent of the red blood cells in 4 days, which lasted for about 6 days, after which a slow and gradual drop in reticulocyte percentage occurred, accompanied by a slow and gradual increase in erythrocytes and hemoglobin. Probably the chief function of Fe in this disease is to stimulate the hematopoietic organs in the bone marrow to produce new reticulocytes above the amount already present.

4. When optimum doses of Cu, Mn, As, Ge, V, or Zn, each, were added separately to 0.25 mg. of Fe, there was no greater degree of reticulocytosis, nor did this process occur quicker, than with this amount of Fe alone, but a big drop in reticulocytes occurred usually on the 5th and sometimes on the 6th day, with a more rapid increase in cells and hemoglobin, than would have been obtained with Fe alone The action of these supplements was therefore to speed up the maturation of the red blood cells.

BIBLIOGRAPHY

1. Minot, G. R., *Tr. College Phys. Philadelphia*, **49**, 144 (1927).
2. Friedlander, A., and Wiedemer, C., *Arch. Int. Med.*, **44**, 209 (1929).
3. Bing, F. C., and Baker, R. W., *J. Biol. Chem.*, **92**, 589 (1931).

4. Levi, A., and Micheletti, A., *Arch. internat. pharmacod.*, **37**, 349 (1930).
5. Chatterjee, B. N., *Indian J. Med. Research*, **16**, 887 (1928).
6. Mettier, S. R., and Minot, G. R., *Am. J. Med. Sc.*, **181**, 25 (1931).
7. Faludi, F., *Orvosi hetil.*, No. 34 (1928), through *Münch. med. Woch.*, **77**, 1506 (1930).
8. Whipple, G. H., *Arch. Int. Med.*, **29**, 711 (1922).

STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

VI. THE EFFECT OF INORGANIC ELEMENTS UPON THE RATE OF BLOOD REGENERATION AND GROWTH

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In Papers I and II of this series of studies, the effectiveness of a given element with Fe upon blood regeneration was determined by the average length of time it required to bring about recovery, as compared with Fe alone under similar experimental conditions. Average results for each group at the beginning and end of the experiment were given. Individual weekly rates of recovery of erythrocytes and hemoglobin were found in many cases to present variations from these averages. Similar variations were found in the increases of body weight.

For the comparison of the results of others in the field of nutritional anemia in the rat with our own, it becomes necessary to make a study of these variations. For instance it may be observed that a small group of anemic rats will show quite marked hemoglobin regeneration the 1st week, with practically complete recovery in 3 weeks. Another group may take a week or 10 days before definite increases in hemoglobin are observed. If those animals which recovered in about 3 weeks had been taken as controls, it would have been necessary to conclude that each of the other elements, when added to Fe, had no better effect than Fe alone, which is obviously not the case.

In this paper will be given the average weekly increase in erythrocytes and hemoglobin, with their percentage variations, the relation between increase in body weight and hemoglobin, and finally a comparison of the growth obtained with Fe, and with Fe plus other elements, in optimum doses. The experimental details were outlined in Papers I and II of this series.

Rate of Erythrocyte and Hemoglobin Regeneration

A group of 76 anemic young rats, which recovered in 6 weeks under a daily dose of 0.5 mg. of Fe, forms the basis of the results to be discussed here. These animals were selected at random from, and are representative of, a much larger group receiving the same amount of Fe under identical conditions.

The relationship between the amount of hemoglobin present and the stage of the treatment with Fe in weeks was measured by calculating the correlation coefficient for these two variables by the product-moment method. A measure of the relationship between the number of erythrocytes and the stage of treatment in weeks was also obtained in a similar manner.

The following coefficients were obtained,

$$r_{\text{Hb per time (6 wks.)}} = 0.95 \pm 0.03$$
$$r_{\text{R.b.c. per time (4 wks.)}} = 0.85 \pm 0.01$$

All conditions of experimentation were the same. The daily dosage of Fe was constant, the daily milk intake was uniform for anemic animals recovering under Fe therapy, and all hemoglobin estimations and cell counts were made by the same worker. Thus we are measuring the relationship between only two variables, and the coefficients obtained are statistically significant.

These correlation coefficients make it possible to obtain prediction equations; the equation in the case of hemoglobin was

$$y = 1.70601x + 3.90130$$

where y = gm. of hemoglobin per 100 cc., and x = weeks. For the erythrocytes,

$$y = 1.22736x + 3.05782$$

where y = erythrocytes, millions per c. mm., and x = weeks.

It is clear from Charts I and II that there is a linear relationship between hemoglobin increase with time and erythrocyte increase with time, under Fe therapy. The zones of probable error of the lines are shown by the dash lines on Charts I and II. The dots on the lines represent the average hemoglobin content and red blood cell counts at the respective weekly intervals. Thus if the experiment has run any number of weeks up to 6 or 4, respectively,
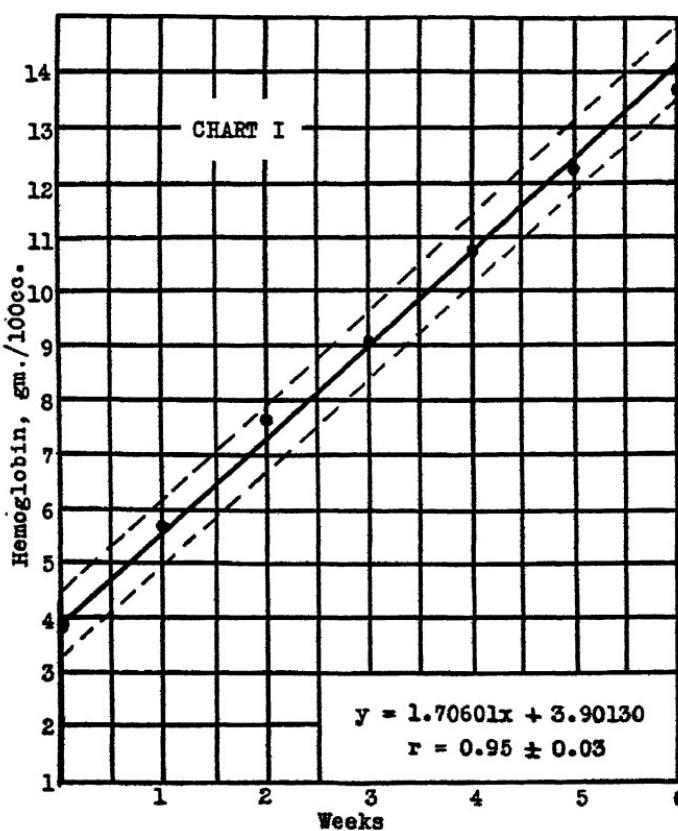
CHART I. Hemoglobin regeneration with time. The zone of probable error is shown by the dash lines. The distribution of observations within and outside of the zone of probable error is as follows:

	Hemoglobin		Erythrocytes	
	Within	Outside	Within	Outside
wks.	*per cent*	*per cent*	*per cent*	*per cent*
0	32.9	67.1	63.2	36.8
1	30.0	70.0	50.0	50.0
2	31.6	68.4	40.8	59.2
3	34.2	65.8	46.0	54.0
4	32.9	67.1	63.0	37.0
5	25.0	75.0		
6	63.5	36.5		

under the conditions of these studies, the average expected hemoglobin content or erythrocyte count can be calculated by the formula.

The chief point of interest in the results presented in Charts I and II is the spread of actual observations in relation to the respective zones of probable error. A normal distribution being assumed, 50 per cent of the observations should come within and 50 per cent outside these zones. By actual count of the 76 cases, it is seen that the variation in hemoglobin regeneration, under the
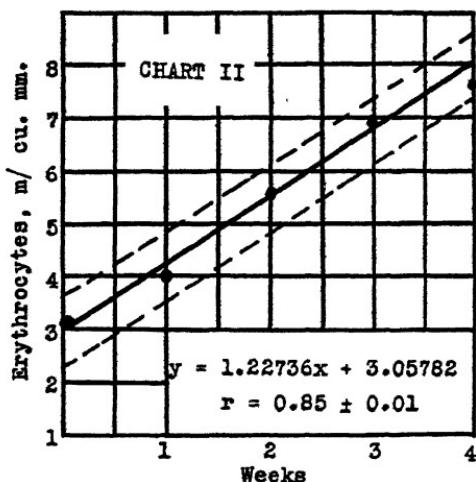
CHART II. Erythrocyte regeneration with time. The zone of probable error is shown by the dash lines and the observations are distributed as given in the legend to Chart I.

experimental conditions here reported, does not follow such a normal distribution. From the beginning of the experiments until the end of 5 weeks, the results for hemoglobin on Fe therapy show that about two-thirds of the cases is outside the zone of probable error, while the remaining one-third is within this zone. At the end of 6 weeks the picture is reversed due to the fact that at this time practically all of the animals have recovered the normal amount of hemoglobin.

In papers, by different investigators, on the effect of inorganic elements on hemoglobin regeneration in the nutritional anemia of

the rat, the results obtained with about three animals are usually reported on a specific dose of element. The total number of animals used is not given. Individual hemoglobin response in these cases, according to the calculations given above, would not correspond to the average results we have presented. It is very likely that two of the animals would show a hemoglobin content falling outside the zone of probable error, while the other would show a content falling within this zone.

Another variable that occurs in this type of investigation, and one that cannot be controlled by the investigator, is the difference in the rate of hemoglobin recovery of anemic rats in different laboratories under identical experimental conditions. Table I gives some interesting comparisons of reported results in these cases.

TABLE I

Comparison of Rate of Hemoglobin Recovery in Nutritional Anemia of Rat As Reported by Different Investigators

Daily dose of Fe	Time for recovery	Daily dose		Time for re-covery	Observer
		Fe	Cu		
mg.	*wks.*	*mg.*	*mg.*	*wks.*	
0.25–0.50	6	0.50	0.05	2–3	Myers and Beard (1)
0.50	No recovery	0.50	0.05	3	Hart, Steenbock, *et al.* (2)
0.40	4	0.50	0.05	8	Mitchell and Schmidt (3)
0.50	No recovery	0.50	0.05	2	Krauss (4)
0.50	9	0.50	0.05	4	Keil and Nelson (5)
0.50	No recovery	0.50	0.10	3	McGhee and coworkers (6)
0.50	" "	0.50	0.05	2–3	Lewis " " (7)

The results given in Table I show that variable rates of hemoglobin recovery under Fe therapy are to be expected. The writer believes that this is due to the following factors: (1) differences in initial hemoglobin concentration (as also emphasized by Mitchell and Miller (8)), (2) differences in the utilization of the Fe, and (3) differences in daily inorganic supplement and milk intake below the optimum necessary for recovery. These facts, with those listed above, make the results of different workers in the field of nutritional anemia of the rat not strictly comparable as far as the time of recovery under Fe therapy is concerned.

The erythrocyte response is much more uniform than that of

the hemoglobin, but since very few, if any, investigators take the trouble to make red blood cell counts in the anemic rats, it is impossible to utilize this factor in comparing the results of others with the data here presented.

Relationship between Increase in Hemoglobin and Body Weight

The correlation coefficients between these two variables at the end of 3 and 6 weeks were calculated.   The results obtained were

$$r_{\text{Hb per body weight (3 wks.)}} = 0.49 \pm 0.06$$
$$r_{\text{Hb per body weight (6 wks.)}} = 0.29 \pm 0.01$$
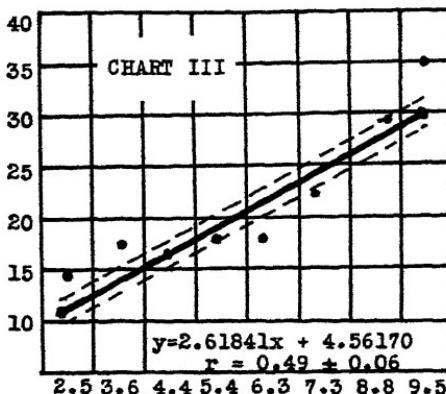
CHART III.   The relationship of body weight to hemoglobin.   The zone of probable error is shown by the dash lines.   The average increase in body weight (ordinate) is plotted in gm. against the average increase of hemoglobin (abscissa) measured in gm. per 100 cc.   The hemoglobin readings are listed along the bottom of the graph.

The first coefficient shows a fair relation between increase in body weight and hemoglobin, and indicates that these two processes take place at the same time (see Chart III).   There is a better relationship at the end of the first 3 weeks than at the end of the second 3 weeks, due to the fact that hemoglobin regeneration is much faster at the beginning of the experiment than at the end, while there is a more or less constant increase in body weight over the whole period.

   The prediction equation calculated from the first coefficient was found to be

$$y = 2.61841x + 4.56170$$

where y = body weight in gm. and x = hemoglobin, gm. per 100 cc. The probable error of the line is ± 0.94 gm. of body weight.

In all cases where the growth of an experimental animal is observed the food intake is a factor of prime importance to the correct interpretation of the results obtained. While we did not measure the milk intake of all of the 750 young rats employed in this series of studies, we did measure the daily intake for the thirty or more animals reported in the reticulocyte study. In general the results show that an anemic young rat after the addition of the inorganic elements to the milk, will take about 25 cc. of milk daily for the 1st week, gradually increasing up to 50 cc. the 2nd week. From this time on until complete recovery takes place, between 50 and 75 cc., or in some cases 100 cc., of milk will be consumed. The writer believes that an adequate milk intake is of equal importance in blood regeneration to the adequate intake of inorganic supplements. We have never observed good blood regeneration where the milk intake has not been adequate. On the other hand, good growth may be obtained without much hemoglobin recovery.

Effect of Inorganic Supplements with Iron upon Growth in Rats Recovering from Nutritional Anemia

It is well known that several of the mineral elements, *e.g.* Fe, I, Ca, Na, K, P, Mn, Mg, Zn, etc., are probably necessary in the physiological economy of the animal organism. Many other elements are known to occur in the body but whether they have any important physiological functions, other than the catalytic effect of some of them upon growth and blood regeneration with Fe in nutritional anemia of the rat, is not known at the present time.

Wright and Papish (9) have recently reported that the spectrographic analysis of the ash of milk shows the presence of "traces" of the following elements, heretofore not known to occur in milk— Se, B, Ti, V, Rb, Li, and Sr. The question then arises as to whether these traces can supplement the Fe additions to the milk, so that recovery may take place, or whether they are present in such small concentration that they are not absorbed or available for blood regeneration. Most investigators do not consider the fact that there is a certain *minimum daily dose* of an element which
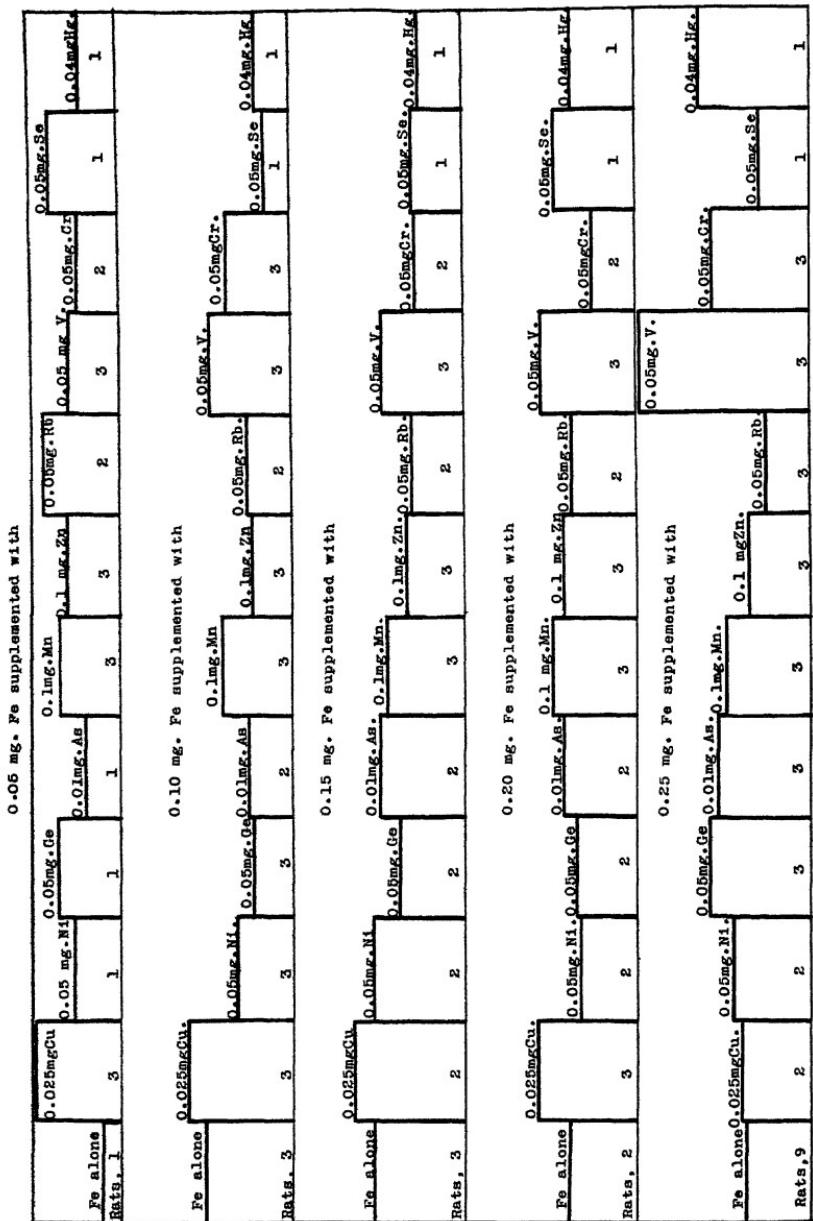
Experimental Period 3 Weeks

(Complete Blood Regeneration in all Cases with Exception of Fe)

Rats 75

Fe alone	Fe + Cu	Fe + Tl	Fe + Ni	Fe + Se	Fe + As	Fe + Ge	Fe + Zn	Fe + Mn	Fe + Cr	Fe + V	Fe + Rb	Fe + Hg
	11	8	13	6	12	23	10	11	6	6	5	9

Influence of Unfavorable Doses of Inorganic Elements (with 0.5 mg. Fe) upon Increase in Body Weight

Experimental Period 6 Weeks

(Blood Regeneration no quicker than on 0.5 mg. Fe alone)

Rats 75

Fe alone	Fe + Cu	Fe + Zn	Fe + Mg	Fe + Al	Fe + Ni	Fe + As	Fe + Mn	Fe + Se	Fe + Rb	Fe + Cr	Fe + Hg	Fe + V
	33	9	11	32	14	18	8	5	4	5	10	5

will be effective with Fe, and that doses below this minimum will have absolutely no effect on blood regeneration, the Fe effect alone being observed in these cases.

Not only do optimum doses of many elements hasten blood regeneration with 0.5 mg. of Fe, but they increase the rate of growth as well. For the convenience of the reader it was thought best to attempt to correlate the growth data obtained with the large number of animals in these studies in the form of charts. This will give us comparative growth data at a glance. The body weight of each animal was taken at the beginning of the experiment and at the end of 3 and 6 weeks. In other cases the weight was taken when complete recovery had taken place from say 2 to 6 weeks.

. In Chart IV will be found a comparison of the growth obtained with Fe alone, and Fe supplemented with optimum doses of other elements for the first 3 weeks of the experiments with the first 331 animals. The average increase in body weight of each group in gm. is given. A marked effect upon growth was obtained with Fe, when the optimum dose of either Cu, Ti, Ni, or Se was added, and a somewhat smaller effect when the other elements were given. Blood regeneration was complete in all cases with the exception of the Fe group, since it takes 6 weeks for average hemoglobin recovery with this element alone.

Chart IV also shows the comparative effect upon growth of 169 anemic young rats when ineffective or unfavorable doses of these elements with 0.5 mg. of Fe were given. Blood regeneration in all these experiments was due to Fe alone. Cu and Zn gave slightly better growth than Fe alone, while Mg gave the best growth of all, an average increase of about 20 gm. This is all the more surprising since this substance occurs in fairly large amounts in the milk and has no effect on blood regeneration. This fact serves to emphasize again the difference between the effect of an element present in a food like milk and when fed as an inorganic salt in slightly acid solution in the milk. All of the remaining elements, Ni to V, gave *less* growth than Fe alone. Thus unfavorable doses will slow up the growth rate.

Further evidence of the parallelism between hemoglobin regeneration and growth will be seen from the data given in Chart V, which shows the average increase in body weight when each of

0.05 mg. Fe supplemented with

| Fe alone Rats, 1 | 0.025mgCu 3 | 0.05 mg.Ni 1 | 0.05mg.Ge 1 | 0.01mg.As 1 | 0.1mg.Mn 3 | 0.1 mg.Zn 3 | 0.05mg.Rb 2 | 0.05 mg.V 3 | 0.05mg.Cr 2 | 0.05mg.Se 1 | 0.04mg.Hg 1 |

0.10 mg. Fe supplemented with

| Fe alone Rats, 3 | 0.025mgCu. 3 | 0.05mg.Ni 3 | 0.05mg.Ge 3 | 0.01mg.As 2 | 0.1mg.Mn 3 | 0.1mg.Zn 3 | 0.05mg.Rb. 2 | 0.05mg.V. 3 | 0.05mgCr. 3 | 0.05mg.Se 1 | 0.04mg.Hg 1 |

0.15 mg. Fe supplemented with

| Fe alone Rats, 3 | 0.025mgCu. 2 | 0.05mg.Ni 2 | 0.05mg.Ge 2 | 0.01mg.As 2 | 0.1mg.Mn. 2 | 0.1mg.Zn. 3 | 0.05mg.Rb. 2 | 0.05mg.V. 3 | 0.05mgCr. 2 | 0.05mg.Se. 1 | 0.04mg.Hg 1 |

0.20 mg. Fe supplemented with

| Fe alone Rats, 2 | 0.025mgCu. 3 | 0.05mg.Ni 2 | 0.05mg.Ge 2 | 0.01mg.As 2 | 0.1 mg.Mn. 3 | 0.1 mg.Zn 3 | 0.05mg.Rb. 2 | 0.05mg.V. 3 | 0.05mgCr. 2 | 0.05mg.Se. 1 | 0.04mg.Hg 1 |

0.25 mg. Fe supplemented with

| Fe alone Rats,9 | 0.025mgCu. 2 | 0.05mg.Ni 2 | 0.05mg.Ge 3 | 0.01mg.As 3 | 0.1mg.Mn. 3 | 0.1 mgZn. 3 | 0.05mg.Rb. 3 | 0.05mg.V. | 0.05mg.Cr. 2 | 0.05mg.Se 1 | 0.04mg.Hg 1 |

Average increase in body weight of group in gm. = weeks of experiment.

CHART V.   Average increase in body weight with optimum doses of elements with doses of Fe from 0.05 to 0.25 mg. daily

the elements supplemented Fe, which was fed in doses from 0.05 to 0.25 mg. daily (for data relating to blood regeneration in these cases see Paper II of this series).

For the convenience of the reader the following hemoglobin regeneration and growth comparisons obtained from Chart V will be given here.

Daily dose of Fe mg.	Hemoglobin regeneration	Increase in body weight
0.05	Cu > As > Mn > Cr	Cu > Rb> Mn > Ge
0.10	Cu > Cr > Mn > As	Cu > V > Mn > Cr
0.15	V > Cu > Mn > As	Cu > Ni > V > As
0.20	V > Cu > Cr > Ni	Cu > V > Mn > Zn
0.25	Cr > Rb > Ge > V	V > Ge> Cr > As

It is easily seen that Cu gave the best hemoglobin regeneration and growth with doses of 0.05 to 0.15 mg. of Fe daily, followed closely by V, Mn, As, and Ge. Ge, V, and Cr, with 0.25 mg. of Fe, gave better growth than Cu. Thus, somewhat the same differentiation of these elements upon growth is obtained as was pointed out in blood regeneration, which again shows the close correlation between these two processes in recovery from nutritional anemia.

SUMMARY AND CONCLUSIONS

Data are presented in this paper covering the following points: (1) the weekly variations in hemoglobin and erythrocyte recovery under Fe therapy, (2) the relationship between increase in hemoglobin and increase in body weight, and finally (3) a comparison of the effect of favorable and unfavorable doses of other elements, with Fe, upon the increase in body weight of anemic rats. The results show:

1. A linear relationship exists between the average hemoglobin content and the duration of the recovery period, expressed in weeks. The same is true for the erythrocyte recovery.

2. Individual weekly determinations for hemoglobin and erythrocytes varied from the average weekly results for these constituents obtained with a group of 76 anemic young rats.

3. A good relationship exists between the increase in hemoglobin and body weight. These two processes take place simultaneously in anemic young rats under Fe therapy during the first

3 weeks and may represent different aspects of the same growth process.

4. When 0.5 mg. of Fe is supplemented with optimum doses of other elements, better growth is secured than with Fe alone, during the same length of time. Whether these mineral supplements actually play a rôle in the growth process, or exert their influence indirectly due to the increased metabolism resulting from the increased hemoglobin formation, is difficult to say.

5. When 0.5 mg. of Fe was supplemented with unfavorable or ineffective doses of other elements, less growth was obtained than with Fe alone (with three or four exceptions).

I wish to acknowledge my indebtedness to Doctor G. E. Harmon of the Department of Hygiene and Bacteriology for suggestions concerning statistical methods used.

BIBLIOGRAPHY

1. Myers, V. C., and Beard, H. H., *J. Biol. Chem.*, **94**, 89 (1931).
2. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., *J. Biol. Chem.*, **77**, 797 (1928).
3. Mitchell, H. S., and Schmidt, L., *J. Biol. Chem.*, **70**, 471 (1926).
4. Krauss, W. E., *J. Biol. Chem.*, **90**, 267 (1931).
5. Keil, H. L., and Nelson, V. E., *Proc. Soc. Exp. Biol. and Med.*, **28**, 392 (1931).
6. Lewis, G. T., Weichselbaum, T. E., and McGhee, J. L., *Proc. Soc. Exp. Biol. and Med.*, **27**, 329 (1930).
7. Underhill, F. A., Orten, J. M., and Lewis, R. C., *J. Biol. Chem.*, **91**, 13 (1931).
8. Mitchell, H. S., and Miller, L., *J. Biol. Chem.*, **92**, 421 (1931).
9. Wright, N. C., and Papish, J., *Science*, **69**, 78 (1929).

THE CARBOHYDRATE ACID SULFATE OF MACROCYSTIS PYRIFERA

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Sulfuric acid esters of carbohydrates are known to occur in mucins and mucoids of animal tissues (1) and in the mucilaginous aqueous extract of a number of algæ (2–8). Although their occurrence in land plants has not been established, they may be constituents of certain plant tissues which contain sulfur in unknown forms of combination (9). Little is known of the composition of the esters of marine plant origin and the constitution of none has been determined. They merit further investigation.

Hoagland and Lieb (2) and Haas (3) and coworkers (6–8) have isolated the calcium salt of acid carbohydrate sulfates from the aqueous extract of various algæ. Haas considers the esters to be disulfates of the type
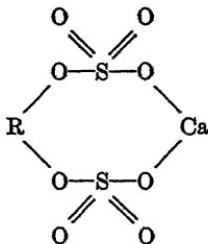
where R represents a polysaccharide residue. Insufficient analytical data were obtained to indicate the composition of the esters. His data do not preclude the presence of polymers of monosulfate carbohydrate esters.

The object of this research was to determine the composition of the ester isolated by Hoagland and Lieb (2) from *Macrocystis pyrifera*. These authors studied a water-soluble substance which

was precipitated with alcohol. After several precipitations from an acid solution and dialysis to remove water-soluble salts, they obtained a substance containing carbohydrate and CaSO₄ "in organic combination" because the sulfate was not precipitated in the cold by BaCl₂. After hydrolysis, tests for sulfate ion and reducing sugar were obtained. The ash was pure CaSO₄ and amounted to 35 per cent of the weight of the sample. The presence of a methylpentose in the preparation was proved by the formation of methylfurfural on boiling with hydrochloric acid. A phenylosazone of the sugar obtained by hydrolysis melted at 172–173° and was considered to be fucose phenylosazone, for which Mayer and Tollens (10) report the m.p. 177.5°. No sugar was isolated.

Our work on the carbohydrate ester from *Macrocystis* has been limited to a preparation obtained by extraction of the air-dried fronds with cold 2 per cent hydrochloric acid and several precipitations with alcohol. The extraction was made with dilute acid because the filtration of the acid solution was more rapid than of the neutral solution and the product obtained on the addition of alcohol was not so gummy. The product was not dialyzed and for this reason contained a large amount of water-soluble inorganic sulfates. We desired to establish the identity of the carbohydrate constituents of the ester and the relative amounts of carbohydrate and sulfuric acid in the ester. We did not consider that the presence of fucose had been established satisfactorily by Hoagland and Lieb by the preparation of an osazone (the sugar might have been the epimer of fucose). No estimation of methylpentose had been reported.

We have isolated and estimated fucose in our preparation and concluded that the ester is a methylpentose monosulfate polymer.

Composition of the Ester—The following analyses indicate the composition of our preparation (see experimental part for details).

	per cent
Inorganic sulfates (ash)	43.8
Methylpentose (as fucose)	31.7
Titratable sulfate as HSO₄⁻	17.6
Uronic acid polymer	2.6
	95.7

The marked acidity of the preparation indicated the presence of an acid ester, the estimation of which might be accomplished without removal of the salts (presumably all inorganic). Accordingly, samples were analyzed for acidity (calculated as HSO_4^-) and methylpentose (calculated as fucose). Acid sulfate and fucose were found to be present in calculated amounts for a fucose monosulfate.

$$\text{Fucose} = \frac{0.317}{164} = 0.00193 \text{ equivalent per gm. (31.7 per cent fucose)}$$

$$HSO_4^- = \frac{0.176}{97} = 0.00181 \text{ equivalent per gm. (17.6 per cent } HSO_4^- \text{ by titration)}$$

The assumption that the titratable acid is acid sulfate is justified by sulfate analyses. The presence of no other sulfate derivative than inorganic sulfates and acid ester being assumed, the sum of the ash sulfate and acid sulfate should equal the total sulfate, and this is seen to be the case.

	per cent SO_4
Ash	27.6
Acid sulfate ester	17.4
	45.0
Total	44.1

The presence of salts of an acid ester is precluded by this agreement since such salts would yield only half of their sulfate as ash sulfate.

On titration with iodine in alkaline solution it was found that only 23.8 per cent of the calculated amount of iodine for the methylpentose was used.

The ester is therefore a methylpentose acid monosulfate polymer of the most probable composition $(R\text{-}O\text{-}SO_2\text{-}OH)_4$.

The isolation of the ester of the monosaccharide by hydrolysis of the polymer may be attended with some difficulty because of the ease with which the sulfuric acid group is hydrolyzed. This difficulty is experienced in work on the carbohydrate esters of mucoproteins (1). Work on this problem is in progress in this laboratory.

The inorganic salts (entirely sulfates) are undoubtedly present as such in the plant.

The uronic acid is probably due to the precipitation of a uronic acid polymer from aqueous solution on the addition of alcohol. An appreciable amount of a uronic acid polymer is extracted from the plant with water. We (11) have previously stated that alginic acid is slightly soluble in water. It is possible that the uronic acid reported by Bird and Haas (8) is not a constituent of an ester, but is likewise due to the precipitation of a uronic acid polymer which would probably not be removed by subsequent dialysis. In this connection it is permissible to suggest that the indications of pentose in the aqueous extract of many algæ may have been due to the presence of acid polymers which are hydrolyzed to uronic acid and lose CO_2 with the formation of a pentose or a substance which exhibits the color reactions of a pentose.

Occurrence of the Ester in the Plant—The presence of calcium, magnesium, and alkali metals in the ash of esters isolated by Haas and coworkers indicates that the esters occur in plants as salts of these metals. It is apparent that the salt of the ester isolated by Bird and Haas (8) from *Laminaria* sp. contains a large amount of metal other than calcium, since the sulfate reported in their ash is far too low for pure calcium sulfate.

Since it is common practice in the isolation of fucose from seaweeds, following the procedure of Günther and Tollens (12) who first isolated fucose, to make a preliminary extraction with cold dilute acid under the conditions used by us for the isolation of the ester, it is apparent that a valuable source of fucose has been discarded. Most of the fucosan in *Macrocystis* (and possibly the algæ which have been used for the isolation of fucose) is present in the first extract. This point was proved by making successive extractions of the ground fronds with cold water, hot water, and hot 2 per cent sulfuric acid and evaporating the extracts to a small volume. A strongly positive test for methylpentose (Rosenthaler's test) was observed in the cold extract, but a negative test was obtained in the following extracts. Incidentally, positive tests for uronic acid (naphthoresorcinol) and pentose (possibly due to uronic acid) were observed in the cold aqueous extract.

<div align="center">EXPERIMENTAL</div>

Isolation of the Ester—1000 gm. of air-dried crushed fronds were extracted with 4000 cc. of 2 per cent hydrochloric acid at room

temperature for 48 hours. The liquid was decanted, pressed from the fronds, and filtered. The filtrate was poured into 3 volumes of 90 per cent alcohol and the precipitate was allowed to settle. The mother liquor was removed with a siphon and the precipitate was washed with alcohol and filtered. Solution in 2 per cent hydrochloric acid and precipitation with alcohol were repeated twice. On repeated precipitation it became necessary to use a centrifuge for complete separation of the substance. It was necessary to filter each solution in order to remove an appreciable amount of $CaSO_4$. The air-dried product weighed 18.9 gm.

Properties and Composition—The product was not dialyzed; hence it contained a large amount of inorganic sulfate precipitated with alcohol. The presence of esterified sulfate was demonstrated, however, by adding an excess of $BaCl_2$ to the hot aqueous solution, filtering, and boiling the clear filtrate acidified with hydrochloric acid, whereupon a precipitate of $BaSO_4$ was obtained. An aqueous solution was distinctly acid and was not viscous even in a concentrated solution as was the original extract of the fronds. A search for inorganic constituents revealed the presence only of calcium and potassium (trace of iron group) among the metals and sulfate (trace of phosphate) among the acids. Chlorides were absent.

The substance did not reduce Fehling's solution in the cold, but became reducing when heated for a short time. Tests on the hydrolyzed substance indicated the presence of methylpentose (HCl and acetone) and the absence of pentose (orcinol), galactose (mucic acid), and glucose, fructose, and mannose (phenylosazone). A negative test for uronic acid (naphthoresorcinol) was obtained, but when the substance was hydrolyzed and a barium salt was precipitated with alcohol, a positive test was obtained on the salt.

Analyses—The ash was determined after ignition with sulfuric acid. The ash so determined was somewhat higher than by ignition without sulfuric acid, presumably because of reduction of sulfate in the latter case and not because of the presence of salts other than sulfate with the ester. Ash and ash sulfate (as $BaSO_4$) were determined in one sample and calcium (as CaO) in another.

The difference between ash and $CaSO_4$ was estimated as "other sulfates" and was found to be mainly potassium sulfate.

Sample	Ash	BaSO₄	CaO	Ash	Ash SO₄	CaSO₄	Other sulfates
gm.	*gm.*	*gm*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*
0.4162	0.1823	0.2792		43.8	27.6		
0.8166			0 0451			13.4	30.4

Total sulfur was determined as $BaSO_4$ after fusion with Na_2O_2 and after hydrolysis with hydrochloric acid. The results were concordant and show that all the sulfur is present as sulfate. After fusion with Na_2O_2 0.1782 gm. yielded 0.1907 gm. of $BaSO_4$. Calculated SO_4 is 44.1 per cent.

0.2540 gm. required 4.22 cc. of 0.1181 N NaOH to an end-point with phenolphthalein. Considering the acidity to be due to an acid sulfate ester and a uronic acid polymer (2.6 per cent of the latter) there is 17.6 per cent HSO_4^- or 17.4 per cent SO_4 due to titratable sulfate.

Methylpentose was estimated by the method of Mayer and Tollens (cited in (13)). 0.2948 gm. yielded 0.0438 gm. of methylfurfural phloroglucide equivalent to 0.0936 gm. of fucose. Found 31.7 per cent fucose.

Uronic acid was estimated by the method of Dickson, Otterson, and Link (14). 0.5562 gm. yielded 0.0036 gm. of CO_2 equivalent to 2.60 per cent uronic anhydride.

0.506 gm. (containing 0.1604 gm. of fucose) was found to be equivalent to 9.37 cc. of 0.0497 N iodine on titration in alkaline solution; calculated 39.3 cc. Hence, 23.8 per cent of the calculated amount of iodine was used.

Isolation of Fucose—2.5 gm. of the substance were boiled under a reflux for 3 hours with 20 cc. of 2.5 per cent sulfuric acid. Sulfuric acid was removed with barium carbonate and the solution was evaporated under reduced pressure to about 10 cc. Salts were precipitated with alcohol. The alcoholic filtrate was evaporated under reduced pressure to about 5 cc. and several volumes of absolute alcohol were added. A sticky white precipitate was obtained. The mother liquor was decanted and evaporated. The syrupy residue gave a positive test for methylpentose with hydrochloric acid and acetone. The syrup was dissolved in 2 to

3 cc. of water and 10 drops of phenylhydrazine were added. Crystallization was induced by scratching. The phenylhydrazone was filtered and washed with water (m.p. 163–164°). Upon recrystallization from 95 per cent alcohol the m.p. was 168–169°. Yield 0.1075 gm. Van der Haar ((13) p. 148) gives 170° as the melting point of fucose phenylhydrazone. The phenylhydrazone was heated in a hot water bath for 40 minutes with 2.5 cc. of water and 2 drops of benzaldehyde. Benzaldehyde phenylhydrazone was separated from the solution by filtration and the solution of the sugar was extracted with ether and evaporated under reduced pressure to a thick syrup. Several cc. of absolute alcohol were added to the syrup and again it was evaporated. The sugar crystallized after standing in a cooler for 2 days. It was carefully washed with several drops of absolute alcohol and several times with larger amounts of absolute ether. The yield of the dry white product was about 0.040 gm. 0.0339 gm. in 3.017 cc. of aqueous solution gave after several hours a constant rotation of $-0.85°$ in a 1 dm. tube at 25°. $[\alpha]_D^{25} = -75.6°$. The most recent and apparently most carefully determined value is $[\alpha]_D^{20} = -76.4°$ (15).

SUMMARY

The acid ester obtained by extraction of the fronds of *Macrocystis pyrifera* with dilute hydrochloric acid is a methylpentose monosulfate polymer of the most probable composition $(R-O-SO_2-OH)_4$. Fucose has been identified as a product of hydrolysis of the ester and is probably the only methylpentose constituent.

BIBLIOGRAPHY

1. Levene, P. A., Hexosamines and mucoproteins, London and New York (1925).
2. Hoagland, D. R., and Lieb, L. L., *J. Biol. Chem.*, **23**, 287 (1915).
3. Haas, P., *Biochem. J.*, **15**, 469 (1921).
4. Neuberg, C., and Ohle, H., *Biochem. Z.*, **125**, 311 (1921).
5. Samec and Ssajevic, V., *Compt. rend. Acad.*, **173**, 1474 (1921).
6. Russell-Wells, B., *Biochem. J.*, **16**, 578 (1922).
7. Haas, P., and Russell-Wells, B., *Biochem. J.*, **23**, 425 (1929).
8. Bird, G. M., and Haas, P., *Biochem. J.*, **25**, 403 (1931).
9. Czapek, F., Biochemie der Pflanzen, Jena, 3rd edition, **2**, 380, 412, 419, 448 (1925).
10. Mayer, W., and Tollens, B., *Ber. chem. Ges.*, **38**, 3021 (1905).

11. Nelson, W. L., and Cretcher, L. H., *J. Am. Chem. Soc.*, **51**, 1914 (1929).
12. Günther, A., and Tollens, B., *Ann. Chem.*, **271**, 86 (1892).
13. van der Haar, A. W., Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin, 83 (1920).
14. Dickson, A. D., Otterson, H., and Link, P. K., *J. Am. Chem. Soc.*, **52**, 775 (1930).
15. Minsaas, J., *Rec. trav. chim. Pays-Bas*, **50**, 424 (1931).

FEEDING EXPERIMENTS WITH MIXTURES OF HIGHLY PURIFIED AMINO ACIDS

I. THE INADEQUACY OF DIETS CONTAINING NINETEEN AMINO ACIDS*†

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Of the twenty amino acids known to be present in proteins only lysine, tryptophane, cystine, and histidine have been definitely established as indispensable dietary components. The importance of eight others, namely glycine, aspartic acid, glutamic acid, hydroxyglutamic acid, proline, hydroxyproline, tyrosine, and arginine, is at the present time uncertain; but in some cases the evidence indicates that they are not essential. The remaining eight, namely alanine, serine, valine, leucine, isoleucine, norleucine, phenylalanine, and methionine, are still unknown quantities in growth physiology.[1]

It appears that if further information is to be secured regarding the relation of the amino acids to maintenance and growth, either more adequate methods must be devised for the quantitative removal of single components of the proteins, or else one must resort to the use of synthetic mixtures of highly purified amino acids. The latter procedure seemed to us to be the more promis-

[1] Evidence for the above classification of amino acids was outlined recently by the author for the final report of the White House Conference on Child Health and Protection.

ing inasmuch as its use removes all uncertainty as to the composition of the basal diet.   With this objective in view we began, some 7 years ago, to accumulate a large stock of amino acids. During the past 15 months feeding experiments have been under way, the outcome of which will be reported in this and subsequent papers.

The literature records the results of several growth investigations in which mixtures of amino acids were employed as the chief or sole sources of nitrogen in the diets.   Hopkins (1916) reports that animals which received a mixture of cystine, tyrosine, lysine, tryptophane, and histidine as the only nitrogen supply experienced a "remarkably slow loss of weight, and long maintenance of apparent health."   When, however, leucine, valine, alanine, glycine, and glutamic acid were fed in place of the previous five, the losses in weight were rapid, and the animals soon succumbed.   At about the same time Osborne and Mendel (1916) described very briefly the results of similar experiments.   They say (p. 2, foot-note):

"We have attempted to learn whether it would be possible to maintain rats on a non-protein diet with additions of tryptophane alone, or together with cystine, histidine, tyrosine, phenylalanine, proline, and ammonium citrate, or urea.   All such attempts failed, even when the supply of energy in the form of non-protein substances was liberal and the food contained all of the necessary inorganic salts and 'food accessories,' and in addition at least 0.5 per cent of protein, present in the 'protein-free' milk.   On such diets the rats declined just as rapidly as when the amino-acid additions were not made."

In experiments involving the alternate feeding of (a) diets containing six to fifteen amino acids and (b) a nitrogen-free diet (except for the nitrogen present in 28 per cent of "protein-free" milk), Mitchell (1916) succeeded in keeping mice alive for 70 to 98 days.   During these periods the animals showed pronounced losses in weight.   The author states that the alternate feeding induced a better total food consumption than did the administration of the amino acid ration alone.   "However," he adds, "it is probable that in no case was the amino-acid intake sufficiently large to assure a fair test of its adequacy."

In 1922 Abderhalden reported the results of extensive studies involving the feeding of diets containing mixtures of amino acids, glucose, fatty acids, glycerol, and inorganic salts, with and with-

out the addition of yeast. In the absence of the latter the animals rapidly lost weight. With the addition of 0.1 gm. of yeast daily the rats are said to have gained. The author concludes (p. 225): "Bei wachsenden Tieren ergab sich, dass die Bausteinnahrung das *Wachstum* nicht unterhalten kann. Erst bei Zusatz von ganz geringen Mengen von Hefe bzw. von Butter usw. kam das Wachstum in Gang." From the description of the diets it is difficult to understand how the animals acquired adequate amounts of vitamins. Furthermore, the make-up of the amino acid mixture is not clear inasmuch as the author reports that it contained two parts of "Pyrrolidinkarbonsäure" and five parts of "Prolin." The identity of these compounds introduces an element of uncertainty which prevents repetition of the experiments. In addition to proline, the amino acid mixture contained eighteen amino acids including α-aminobutyric acid. Hydroxyglutamic acid was not present; and methionine, at the time of publication of the paper, had not yet been identified as a component of proteins.

More recently Suzuki, Matsuyama, and Hashimoto (1926) attempted to maintain rats upon a ration of purified amino acids, protein-free milk, butter, starch, and calcium lactate and phosphate. Fourteen amino acids and ammonium carbonate were employed as the source of nitrogen. Serine, isoleucine, norleucine, hydroxyglutamic acid, hydroxyproline, and methionine were not included. The animals rapidly lost weight, and died in the course of the experiments.

EXPERIMENTAL

Of the nineteen amino acids used in these investigations thirteen were prepared in this laboratory from their natural sources. The remaining six were synthetic products. All were beautifully crystalline, white compounds. The purity of each was demonstrated by analysis. In the case of the racemic acids, twice the desired amounts were employed in order to insure the presence of the natural enantiomorphs in suitable proportions. Histidine, arginine, and lysine were used in the form of their hydrochlorides, the calculated quantity of sodium bicarbonate necessary to neutralize the hydrochloric acid being added to the mixture.

In formulating our first amino acid mixture we imitated the composition of casein in so far as available information permitted.

In the second column of Table I the figures represent the usually accepted values for the known amino acids in this protein.   How-

TABLE I

Composition of Amino Acid Mixtures

	Casein	Mixture I		Mixture II	
		Active amino acids	As used	Active amino acids	As used
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Glycine...........................	0.45	0.50	0.50	1.00	1.00
Alanine...........................	1.85	1.90	3.80*	2.00	4.00*
Valine............................	7.93	8.00	16.00*	8.00	16.00*
Leucine...........................	9.70†	9.00	9.00	10.00	10.00*
Isoleucine........................	?	1.25	2.50*	1.25	2.50*
Norleucine........................	?	1.25	2.50*	1.25	2.50*
Proline...........................	7.63	8.00	8.00	8.00	8.00
Hydroxyproline...................	0.23	0.30	0.30	4.00	4.00
Phenylalanine.....................	3.88	3.90	7.80*	5.00	10.00*
Glutamic acid.....................	21.77	22.00	22.00	10.00	10.00
Hydroxyglutamic acid.............	10.50	0	0	0	0
Aspartic acid.....................	4.10	4.10	4.10	2.00	2.00
Serine............................	0.50	0.50	1.00*	0	0
Tyrosine..........................	4.50	6.50	6.50	5.00	5.00
Cystine...........................	?	1.25	1.25	3.00	3.00
Histidine.........................	2.50	2.75		5.50	
" hydrochloride..........			3.40		6.80
Arginine..........................	3.81	5.25		5.25	
" hydrochloride...........			6.35		6.35
Lysine............................	7.62	7.70		10.00	
" dihydrochloride...........			11.55		15.00
Tryptophane.......................	1.50	2.25	2.25	4.00	4.00
Sodium bicarbonate...............			12.86		16.99
	88.47	86.40	121.66‡	85.25	127.14§

* Racemic acids.

† Includes isoleucine.

‡ 1.408 gm. of mixture are equivalent to 1.0 gm. of "effective" amino acids.

§ 1.491 gm. of mixture are equivalent to 1.0 gm. of "effective" amino acids.

ever, in the case of a few acids, notably tyrosine, arginine, and tryptophane, somewhat higher values are sometimes encountered

in the literature.   We have employed the larger quantities when the evidence appeared to us to warrant it.   So little is known concerning the proportions of isoleucine, norleucine, and cystine in casein that the values selected by us represent little more than guesses.   All figures were somewhat rounded upward in preparing the mixture.

In the third column of Table I are shown the quantities of natural, free amino acids in our Mixture I.   In the fourth column the figures represent the actual amounts employed after allowing for the hydrochlorides and the double proportions of racemic acids. From the totals of the two columns one may calculate that 1.408 gm. of Mixture I are equivalent to 1.0 gm. of what we have chosen to call the "effective" amino acids.   This factor was made use of in calculating the quantity of the mixture employed in the diet.

The last two columns of Table I show similar data for Mixture II.   This differs from Mixture I primarily in having larger proportions of glycine, hydroxyproline, phenylalanine, cystine, histidine, lysine, and tryptophane.   A few other components are slightly increased.   On the other hand, Mixture II contains less glutamic acid, aspartic acid, and tyrosine than Mixture I, and is devoid of serine.   Our supply of the latter was very limited. Experiments in this laboratory (Bunney and Rose, 1928; St. Julian and Rose, unpublished data) have shown that a very large proportion of the dicarboxylic acids and tyrosine present in hydrolyzed casein may be removed without diminishing the nutritive value of the residual materials.   Thus the effect of the use of Mixture II was to increase the proportions of the amino acids known to be essential, and of certain others about which little or no nutritive information is available, while decreasing some of those which in Mixture I are certainly present in excess of the growth requirements.   It is evident from the data that 1.491 gm. of Mixture II are equivalent to 1.0 gm. of "effective" amino acids. In preparing the mixtures, the amino acids were carefully weighed, thoroughly mixed by hand, and ground several times in a mill. The resulting products were passed through a 60 mesh sieve and bottled.

It will be observed that neither mixture contained hydroxyglutamic acid.   This amino acid was not available to us.   It is generally regarded as a non-essential dietary component inasmuch

as edestin, which presumably is devoid of hydroxyglutamic acid (*cf.* Osborne, Leavenworth, and Nolan, 1924), supports normal growth.   As will be outlined in Paper III of this series, the addition to our amino acid ration of a protein fraction containing hydroxyglutamic acid did not improve the nutritive value of the food.   Thus its absence could not have been a limiting factor in our experiments.   Because of the small supply of methionine available at the beginning of the investigation, this amino acid

TABLE II

*Composition of Diets**

	Diet A	Diet A-1
	gm.	*gm.*
Amino acid Mixture I (18 amino acids).........	23.8	
" " " II (17 " ").........		24.1
Methionine (*l*)................................	0.3	1.0
Glucosamine hydrochloride (*d*).................	1.0	1.0
Sodium bicarbonate...........................	0.4	0.4
Dextrin.......................................	22.5	21.5
Sucrose.......................................	15.0	15.0
Salt mixture†.................................	4.0	4.0
Agar..	2.0	2.0
Lard..	26.0	26.0
Cod liver oil..................................	5.0	5.0
	100.0	100.0

* Each diet contained 18 per cent of "effective" amino acids including glucosamine.   The vitamin B factors were supplied in the form of two pills daily, each containing 100 mg. of yeast.

† Osborne and Mendel (1919).

was not incorporated in either mixture, but was introduced directly into the rations at the time the latter were prepared for feeding (*cf.* Table II).

The make-up of the diets is shown in Table II.   As will be observed, Diet A contained 0.3 per cent of *l*-methionine.   In Diet A-1 this was increased to 1.0 per cent.   Both diets contained 1.0 per cent of glucosamine hydrochloride, with 0.4 per cent of sodium bicarbonate to neutralize the hydrochloric acid.   Glucosamine may not be a dietary essential, but the fact that Riming-

ton (1929) and others have found it in certain proteins renders its nutritive status uncertain. In order to exclude the possibility of a deficiency from this source glucosamine was included in the food. Each diet contained an amount of the amino acid mixture necessary to provide 18 per cent of "effective" amino acids including glucosamine. In addition to the latter Diet A carried nineteen amino acids, and Diet A-1 eighteen. Thus the only known protein component absent from Diet A was hydroxyglutamic acid, while both hydroxyglutamic acid and serine were missing from Diet A-1. The only nitrogenous material of unknown nature in the rations was that contained in 200 mg. of yeast fed to each animal daily as a source of the vitamin B factors.[2]

Young white rats were employed as the experimental animals. Because of the enormous expense involved in the use of such diets the experiments were limited to 36 days each. The food was administered *ad libitum*. The growth curves are shown in Charts I and II. It is evident that both diets were totally inadequate to meet the nutritive demands of growth. The animals lost weight at extremely rapid rates during the first 12 days, and then declined gradually or maintained weight to the end of the experiments. No significant differences are to be observed between the rats upon the two diets. Despite the increased proportions of eight amino acids in Diet A-1 the animals which received this food experienced just as profound nutritive failure as did those upon Diet A. Thinking that possibly the absence of serine from Diet A-1 might account for its poor nutritive quality, Rats 1758 and 1759, during the last 8 days of the experiments, received an identical ration except that 2 per cent of *dl*-serine was incorporated in the food in place of a like amount of dextrin. Thus the total proportion of "effective" amino acids was increased to 19 per cent, and the diet then contained nineteen amino acids. This measure also failed to promote the nutritive well being of the animals.

[2] We are aware, of course, that 200 mg. of yeast cannot provide *optimal* amounts of vitamin B factors. Because of the presence in yeast of amino acids it was necessary for us to employ the smallest quantity of this substance which is capable of inducing satisfactory growth. That excellent growth can be attained when otherwise adequate diets are supplemented with 200 mg. of yeast is demonstrated by the charts in Paper III of this series. We have not yet been able to secure or prepare a vitamin B concentrate which is more suitable for our purposes than is yeast.

CHART I.

GROWTH ON DIET A.

19 AMINO ACIDS.

1560♀ (68)

1559♂ (77)

(50)

1558♂ (74)

(56)

1557♂ (75)

(54)

1556♂ (79)

(54)

1555♂ (75)

←10 days→

←10 gms.→

(53)

(54)
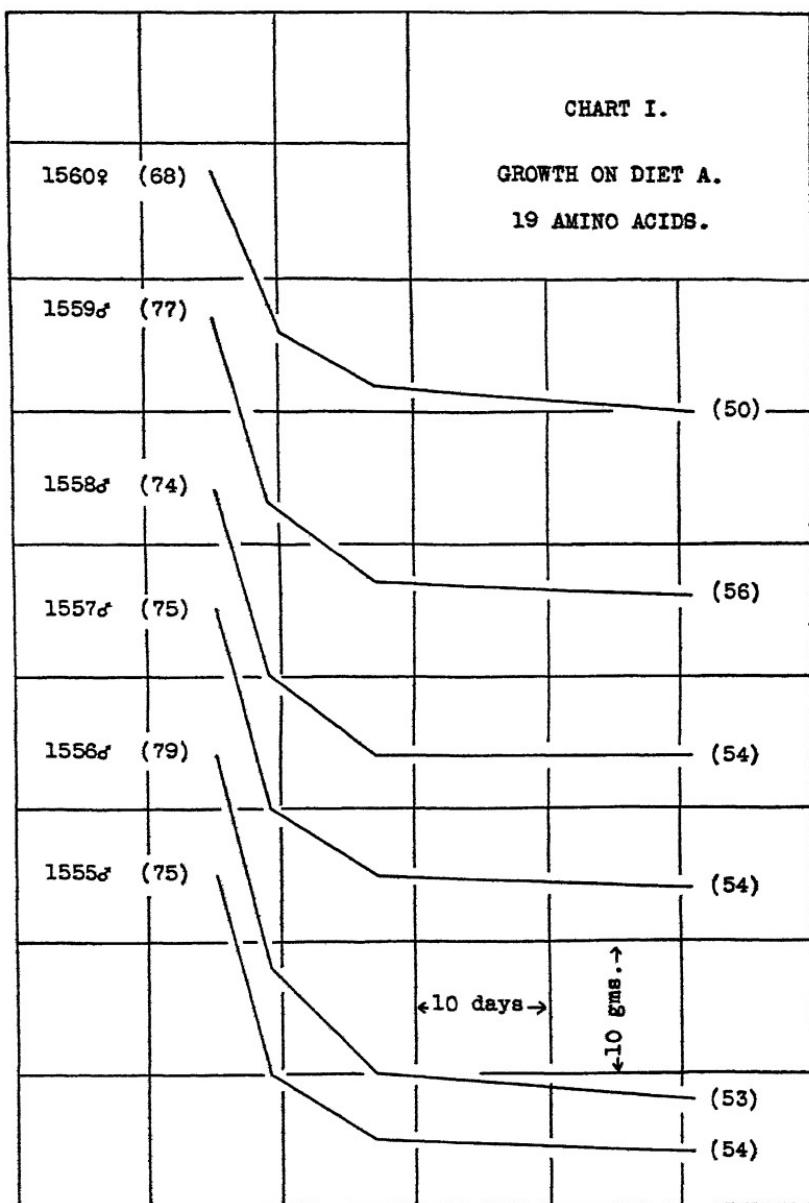
CHART I. The numbers in parentheses denote the initial and final weights of the rats.

In Table III are recorded the figures representing the total changes in body weight and total food intakes of the rats. As was to be expected with diets so completely inadequate for growth

CHART II.
GROWTH ON DIET A-1.
18 AMINO ACIDS.

At arrows, diet of Rats 1758
and 1759 was supplemented
with 2 % dl-serine.

1759♂ (96)

1758♀ (85)

←10 days→

1757♀ (87)

1756♀ (77)

1755♀ (85)

←10 gms.→

(72)

(64)

(65)

(58)

(63)
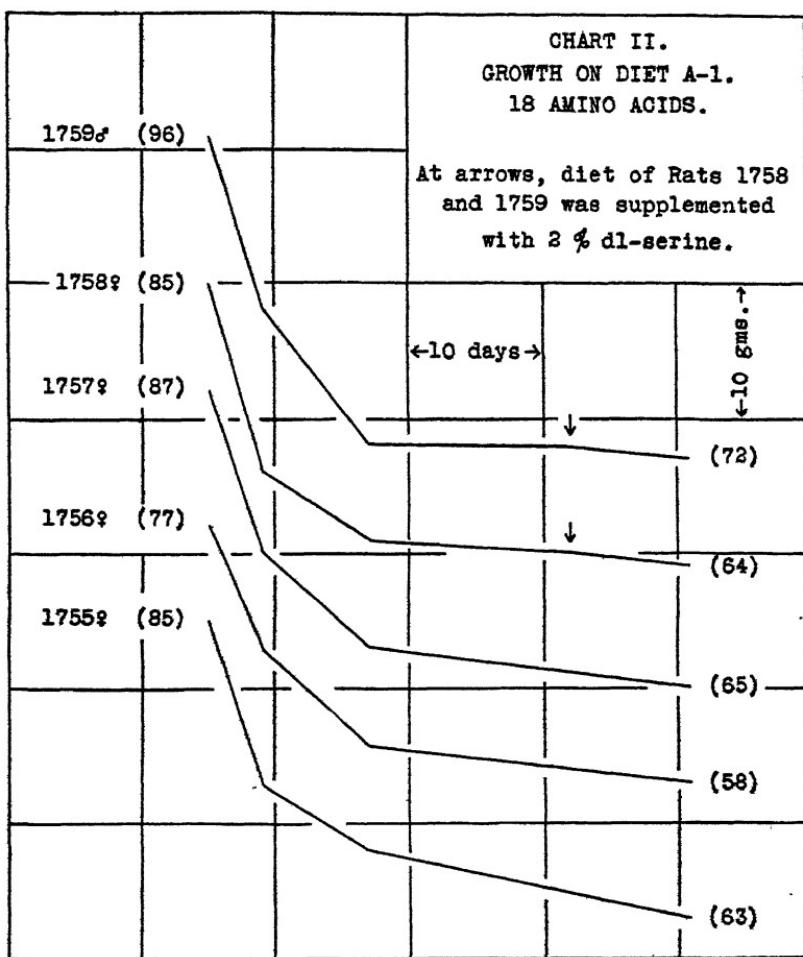
Chart II. The numbers in parentheses denote the initial and final weights of the rats.

purposes, the food intakes are exceedingly low. Our experience with other types of deficiencies involving the nitrogenous portion of the ration has taught us to expect *a marked failure in appetite*

when the diet is completely devoid of an essential component. On the other hand, if the deficiency is only slight the diminution in food consumption may not be very pronounced. Indeed, when the inadequacy is sufficiently small to permit the animals to compensate by the consumption of more food, we have actually observed moderate increases, for a time, in the food intakes.

We were not surprised, therefore, at the results obtained in the present investigation. Nor do we believe that the failure of our animals to consume larger quantities of the diets can be correctly

TABLE III

Total Changes in Body Weight and Total Food Intake

The experiments covered 36 days each.

Rat No. and sex	Diet	Total change in weight	Total food intake
		gm.	*gm.*
1555♂	A	−21	92
1556♂		−26	104
1557♂		−23	91
1558♂		−20	93
1559♂		−21	90
1560♀		−18	100
1755♀	A-1	−22	97
1756♀		−19	74
1757♀		−22	88
1758♀		−21*	95
1759♂		−24*	96

* During the last 8 days the diet of Rats 1758 and 1759 was supplemented with 2 per cent of *dl*-serine which replaced an equal amount of dextrin.

attributed to the taste, consistency, or other more or less superficial characteristics of the food. Hundreds of experiments in this laboratory, involving the use of completely hydrolyzed proteins, have demonstrated that rats readily consume adequate amounts of diets in which the nitrogenous portion is in the form of mixtures of amino acids provided all of the materials necessary for growth are available. These considerations have led us to the conclusion that *growth-promoting proteins either contain at least one essential other than the twenty recognized amino acids, or furnish one or more*

of the known amino acids in far greater proportions than is generally believed. We are of the opinion that the former possibility is the more probable one. In subsequent papers of this series evidence will be presented pointing to the existence in proteins of a hitherto unknown constituent which is indispensable for growth.

SUMMARY

Feeding experiments have been conducted with diets in which the protein (other than that present in 200 mg. of yeast employed daily as a source of the vitamin B factors) has been replaced by mixtures of highly purified amino acids. When all of the well recognized protein components except hydroxyglutamic acid are incorporated in the food, rats receiving such diets rapidly lose weight during the first 12 days, and then decline gradually or maintain weight to the end of the experiments. Inasmuch as the addition of a protein fraction carrying the dicarboxylic amino acids fails to improve the quality of the food, as will be demonstrated in a subsequent paper, it is evident that the absence of hydroxyglutamic acid cannot account for the inability of the animals to increase in weight. The results appear to indicate that growth-promoting proteins contain at least one essential dietary component other than the twenty known amino acids.

BIBLIOGRAPHY

Abderhalden, E., *Arch. ges. Physiol.*, **195**, 199 (1922).
Bunney, W. E., and Rose, W. C., *J. Biol. Chem.*, **76**, 521 (1928).
Hopkins, F. G., *J. Chem. Soc.*, **109**, 629 (1916).
Mitchell, H. H., *J. Biol. Chem.*, **26**, 231 (1916).
Osborne, T. B., Leavenworth, C. S., and Nolan, L. S., *J. Biol. Chem.*, **61**, 309 (1924).
Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **25**, 1 (1916); **37**, 572 (1919).
Rimington, C., *Biochem. J.*, **23**, 430 (1929).
Suzuki, U., Matsuyama, Y., and Hashimoto, N., *Sc. Papers Inst. Physic. and Chem. Research*, Tokyo, **4**, 1 (1926).

FEEDING EXPERIMENTS WITH MIXTURES OF HIGHLY PURIFIED AMINO ACIDS

II. THE SUPPLEMENTING EFFECT OF PROTEINS*

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In the preceding paper the conclusion was reached that growth-promoting proteins probably carry at least one indispensable dietary component other than the twenty generally accepted amino acids, and that the inadequacy of diets containing synthetic mixtures of the known amino acids is due to the absence of this hitherto unrecognized essential. If this conception is correct one should be able to demonstrate at least a moderate improvement in the quality of the food as a result of replacing a portion of the amino acid mixture with an equivalent quantity of protein. In order to test the validity of this hypothesis the following experiments were conducted in which the supplementing action of gelatin, gliadin, and casein was determined.

EXPERIMENTAL

Three diets were formulated, each of which contained 5 per cent of one of the proteins indicated above in place of a like amount of "effective" amino acids. The amino acid mixture was identical in composition with Mixture I described in the preceding paper except that it contained no serine. It will be referred to in this and subsequent papers as Mixture III. Our supply of both

serine and methionine had been exhausted temporarily at the time these experiments were undertaken; but preliminary studies had already shown that the growth rates of rats, under the conditions in question, are not affected by the presence or absence of these two amino acids. This must not be interpreted, of course, as indicating that serine and methionine are not essential, since the proteins used as supplements may have furnished sufficient quantities to meet the needs of the animals.

Each diet had the composition shown in Table I. Inasmuch as 1.405 gm. of Mixture III are equivalent to 1.0 gm. of "effective"

TABLE I

*Composition of Diets**

	gm.
Amino acid Mixture III†	18.3‡
Supplement (gelatin, gliadin, or casein)	5.0
Dextrin	26.7
Sucrose	15.0
Salt mixture§	4.0
Agar	2.0
Lard	24.0
Cod liver oil	5.0
	100.0

* The vitamin B factors were supplied in the form of two pills daily each containing 100 mg. of yeast.

† Identical in composition with Mixture I described in Paper I except that serine was absent.

‡ Equivalent to 13.0 per cent of "effective" amino acids.

§ Osborne and Mendel (1919).

amino acids, it is evident that 18.3 gm. of the mixture provided 13 gm. of "effective" acids. Thus each ration contained 18 per cent of nitrogenous materials in addition to the unnatural enantiomorphs present in the racemic acids. As in the preceding paper, the vitamin B factors were supplied in the form of two pills daily each containing 100 mg. of yeast.

The results of the experiments are summarized in Chart I and Table II. As will be observed, the animals at first lost weight just as if no supplement had been provided. However, the minimal weights were reached in 4 days, after which slow growth en-
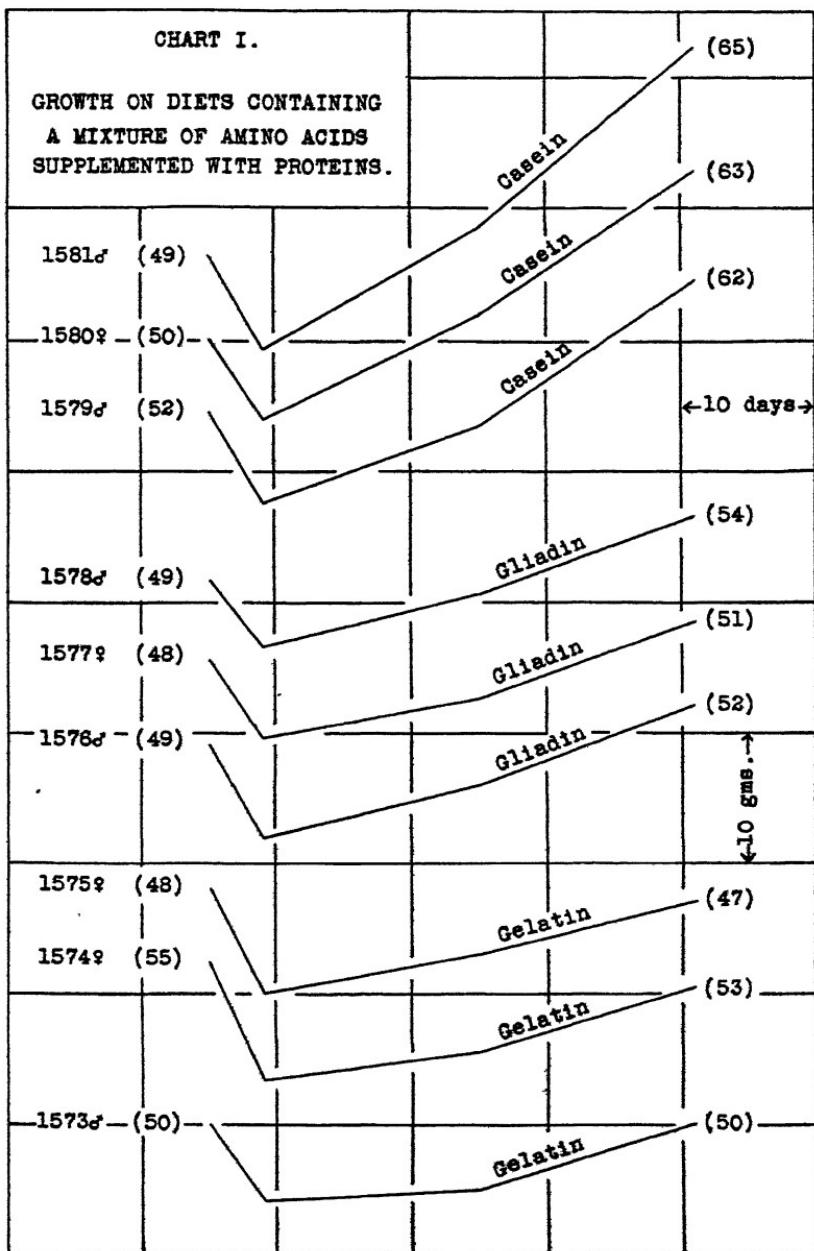
CHART I.

GROWTH ON DIETS CONTAINING
A MIXTURE OF AMINO ACIDS
SUPPLEMENTED WITH PROTEINS.

1581♂   (49)

1580♀ — (50)

1579♂   (52)

1578♂   (49)

1577♀   (48)

1576♂ — (49)

1575♀   (48)

1574♀   (55)

1573♂ — (50)

Casein   (65)

Casein   (63)

Casein   (62)

←10 days→

Gliadin   (54)

Gliadin   (51)

Gliadin —   (52)

←10 gms.→

Gelatin   (47)

Gelatin   (53)

Gelatin   (50)

CHART I. The numbers in parentheses denote the initial and final

sued.   In the case of gelatin, the supplementing effect was scarcely sufficient to enable the rats to regain the losses experienced at the beginning of the experiments.   This is a matter of considerable interest in view of the observation of Jackson, Sommer, and Rose (1928) that the addition to a gelatin diet of the eleven known amino acids, which on the basis of the best analyses available seem to be the most likely possible sources of deficiency in this protein, fails to render the food satisfactory for growth.   Apparently, gelatin contains very little of the growth essential lacking in our amino acid mixtures.   We expect to report further upon this problem in a later paper.

TABLE II

Total Changes in Body Weight and Total Food Intake on Diets Containing a Mixture of Amino Acids Supplemented with 5 Per Cent of Protein

The experiments covered 36 days each.

Rat No. and sex	Supplement	Total change in body weight	Total food intake
		gm.	*gm.*
1573♂	Gelatin	0	93
1574♀	"	−2	110
1575♀	"	−1	90
1576♂	Gliadin	+3	87
1577♀	"	+3	89
1578♂	"	+5	99
1579♂	Casein	+10	103
1580♀	"	+13	107
1581♂	"	+16	114

The supplementing effect of gliadin was only slightly better than that manifested by gelatin.   However, each animal which received this protein attained a somewhat greater body weight than that with which it began the experiment.   With casein the results were more striking.   Upon this ration the rats made total gains of 10 to 16 gm. each.   When one recalls that the animals which received the amino acid diets *without* protein supplementation (Paper I) experienced losses of 18 to 26 gm., it is evident that the proteins in question exerted distinctly beneficial effects.

It has been shown that very small quantities of milk proteins (Osborne and Mendel, 1915, and McCollum and Davis, 1915),

or of the proteins of the wheat kernel (Osborne and Mendel, 1920) permit slow growth. In view of these findings one may ask whether the increases in weight of our animals are not to be ascribed entirely to the proteins *per se* rather than to a supplementing effect exerted by them upon the amino acid mixture. Obviously, this question cannot be answered with certainty from the data available. But inasmuch as the amino acid mixture alone supplied all known protein constituents (other than serine, hydroxyglutamic acid, and methionine, which were not limiting factors), it seems logical to assume that *the supplements furnished something which was lacking in the amino acid mixture.* If so, the active material must be a hitherto unrecognized protein component. Certainly, a supplementing effect appears to be the only satisfactory explanation for the results obtained in the gelatin experiments, since all proportions of this material, when used as the sole source of nitrogen, fail to permit growth. Furthermore, since neither gelatin nor gliadin improved the nutritive quality of the ration as effectively as did casein the latter would appear to be the most promising protein of the three to employ in a search for the unknown growth essential.

SUMMARY

Experiments have been conducted to determine the supplementing effect of proteins when added to diets containing a synthetic mixture of amino acids. The results show that when 5 per cent of casein, gliadin, or gelatin is substituted for an equivalent quantity of the amino acid mixture, the animals lose weight for 4 days and then slowly gain. Casein is more effective than either gliadin or gelatin in stimulating growth. This growth stimulation appears to provide additional evidence for the existence in proteins of an unknown dietary essential.

BIBLIOGRAPHY

Jackson, R. W., Sommer, B. E., and Rose, W. C., *J. Biol. Chem.,* **80,** 167 (1928).
McCollum, E. V., and Davis, M., *J. Biol. Chem.,* **20,** 415 (1915).
Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.,* **20,** 351 (1915); **37,** 572 (1919); **41,** 275 (1920).

FEEDING EXPERIMENTS WITH MIXTURES OF HIGHLY PURIFIED AMINO ACIDS

III. THE SUPPLEMENTING EFFECT OF CASEIN FRACTIONS*

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(Received for publication, August 14, 1931)

Having already secured evidence, we believe, for the existence of a hitherto unrecognized essential in growth-promoting proteins, we have next undertaken the fractionation of casein for the purpose of concentrating the active material. The presence or absence of the growth factor has been demonstrated in each fraction by determining the supplementing effect of the latter when incorporated in a diet containing a mixture of amino acids as the only other source of nitrogen. The methods employed in the conduct of the experiments and the results obtained are described in detail below.

EXPERIMENTAL

For reasons which were explained in the preceding paper, casein was selected as the most promising protein for the purpose in question. The fractionation was carried out as follows: 2 kilos of the protein were refluxed on a sand bath for 16 hours with a mixture of 2500 cc. of concentrated sulfuric acid and 7500 cc. of distilled water. After diluting with 24 liters of water, the mixture was treated with finely powdered barium hydroxide until only a trace of sulfuric acid remained. The precipitate was filtered off and washed twice by suspending in 15 liters of water, and passing in steam until the mixture had been heated to the boiling point. The combined filtrate and washings were then evaporated *in*

vacuo to a volume of approximately 15 liters, and the last traces of sulfuric acid were removed by the cautious addition of a hot saturated solution of barium hydroxide. After again filtering, the filtrate was further concentrated under reduced pressure to a volume of about 3 liters, and was allowed to stand in an ice box for the crystallization of the less soluble amino acids. These were then filtered off and recrystallized once by dissolving in 4 liters of hot water, concentrating to 1500 cc., and allowing to stand on ice for 18 hours. The precipitate (Fraction 1) was removed by filtration and dried. The filtrate was returned to the original solution.

The combined filtrates from the less soluble amino acids were employed for the precipitation of the dicarboxylic amino acids according to the procedure of Kingston and Schryver (1924). For this purpose, barium hydroxide was added in 30 per cent excess (as determined by a Sörensen titration), and the whole was treated with 4 volumes of 95 per cent alcohol. After standing in the ice box for 2 days, the barium salts of the dibasic acids were removed by filtration, suspended in 5 liters of water, and decomposed by the addition of a slight excess of sulfuric acid. The filtrate from the barium sulfate was treated cautiously with a solution of barium hydroxide until neither the barium nor sulfate ion could be detected, filtered, and evaporated to dryness *in vacuo* (Fraction 2).

The filtrate from the dibasic acids was freed from barium by the addition of a slight excess of sulfuric acid, and from alcohol by vacuum distillation. After adding water and sufficient sulfuric acid to make a concentration of 5 per cent the diamino acids were precipitated in the usual fashion with phosphotungstic acid. The total volume at this point amounted to about 10 liters. The solution was allowed to stand in the ice box for 2 days, filtered, and the precipitate was washed with 5 per cent sulfuric acid. The phosphotungstates were then suspended in dilute sulfuric acid, treated with an equal volume of acetone, and extracted with ether for the removal of the phosphotungstic acid. The water-acetone mixture containing the diamino acids was then freed from sulfuric acid and evaporated to dryness *in vacuo* (Fraction 3).

The filtrate from the diamino acids was treated with an excess of barium hydroxide for the removal of the sulfuric and phosphotungstic acids, filtered, and the excess barium exactly precipitated

by the cautious addition of dilute sulfuric acid. The final filtrate was evaporated *in vacuo* to a volume of 2 liters, and was extracted twice with butyl alcohol for the removal of the monoaminomonocarboxylic acids according to the Osborne, Leavenworth, and Nolan (1924) modification of the Dakin (1918) procedure. 10 liters of butyl alcohol were employed in the first extraction, and 1280 cc. in the second. The combined extracts were then concentrated under diminished pressure to a small volume and allowed to cool for the crystallization of the monoamino acids. The latter were filtered off and dried *in vacuo* (Fraction 4). The butyl alcohol filtrate from the monoamino acids was further concentrated to a syrup for the purpose of recovering the crude proline fraction. Unfortunately, during the drying of this sticky material in the vacuum oven a defect in the thermostatic regulator permitted the temperature to rise too high during the night, and this fraction had to be discarded. Therefore, another proline fraction was prepared in exactly the same way from a second lot of casein, and was used in two feeding experiments as will be noted below.

The water residue left after the butyl alcohol extractions was concentrated under diminished pressure. In the course of the evaporation part of the amino acids crystallized out, and was filtered off and dried (Fraction 5). The filtrate was then evaporated to dryness, giving an additional residue (Fraction 6).

Each of the above casein fractions was employed in the crude state without further purification. No attempt was made to recover all of each fraction, and considerable material was lost through adsorption on the bulky precipitates of barium sulfate and barium phosphotungstate. This was not regarded as a matter of importance inasmuch as the present study was a *qualitative* one designed to determine whether the unknown growth essential could be concentrated in a single protein fraction.

The feeding experiments were conducted in a slightly different fashion from those recorded in the preceding paper. Instead of including the supplement in the food from the start, the rats for 12 days were placed upon a ration (Diet B) containing a mixture of eighteen amino acids as the sole source of nitrogen. During this fore period the animals at first lost weight rapidly, and then more gradually. They were then transferred to Diet C containing

5 per cent of one of the casein fractions in place of an equivalent amount of "effective" amino acids.   Inasmuch as 1.405 gm. of Mixture III are equivalent to 1.0 gm. of "effective" acids, the proportion of Mixture III in Diet C was placed at 16.7 per cent. Thus each ration provided 18 per cent of "effective" acids including glucosamine.   As was pointed out in the preceding paper, serine and hydroxyglutamic acid are the only generally recognized protein components which were not introduced as such into the food.

TABLE I

*Composition of Diets**

	Diet B	Diet C
	gm.	*gm.*
Amino acid Mixture III (17 amino acids)	23.8	16.7
Supplement (casein fraction)..................		5.0
Methionine (*dl*)..............................	0.6	0.6
Glucosamine hydrochloride (*d*)................	1.0	1.0
Sodium bicarbonate...........................	0.4	0.4
Dextrin......................................	22.2	25.3
Sucrose......................................	15.0	15.0
Salt mixture†................................	4.0	4.0
Agar..	2.0	2.0
Lard..	26.0	25.0
Cod liver oil.................................	5.0	5.0
	100.0	100.0

 * Each diet contained 18 per cent of "effective" amino acids, including glucosamine.   The vitamin B factors were supplied in the form of two pills daily, each containing 100 mg. of yeast.
 † Osborne and Mendel (1919).

The make-up of the diets is shown in Table I.   In addition to the basal rations, which were fed *ad libitum*, each animal received two pills daily each containing 100 mg. of yeast as a source of vitamin B factors.   The effects of the casein fractions upon the growth of two typical litters of animals are shown in Charts I and II.   In Chart III are presented the growth curves of two rats which received the proline fraction prepared from the second lot of hydrolyzed casein.   In Table II are summarized the total change in weight and total food intake of each of the twenty-four rats which received the supplements described above.   From the data

CHART I.

GROWTH ON DIET B (18 AMINO ACIDS),
AND ON DIET C (B SUPPLEMENTED WITH
CASEIN FRACTIONS).

Supplements were introduced at
arrows.
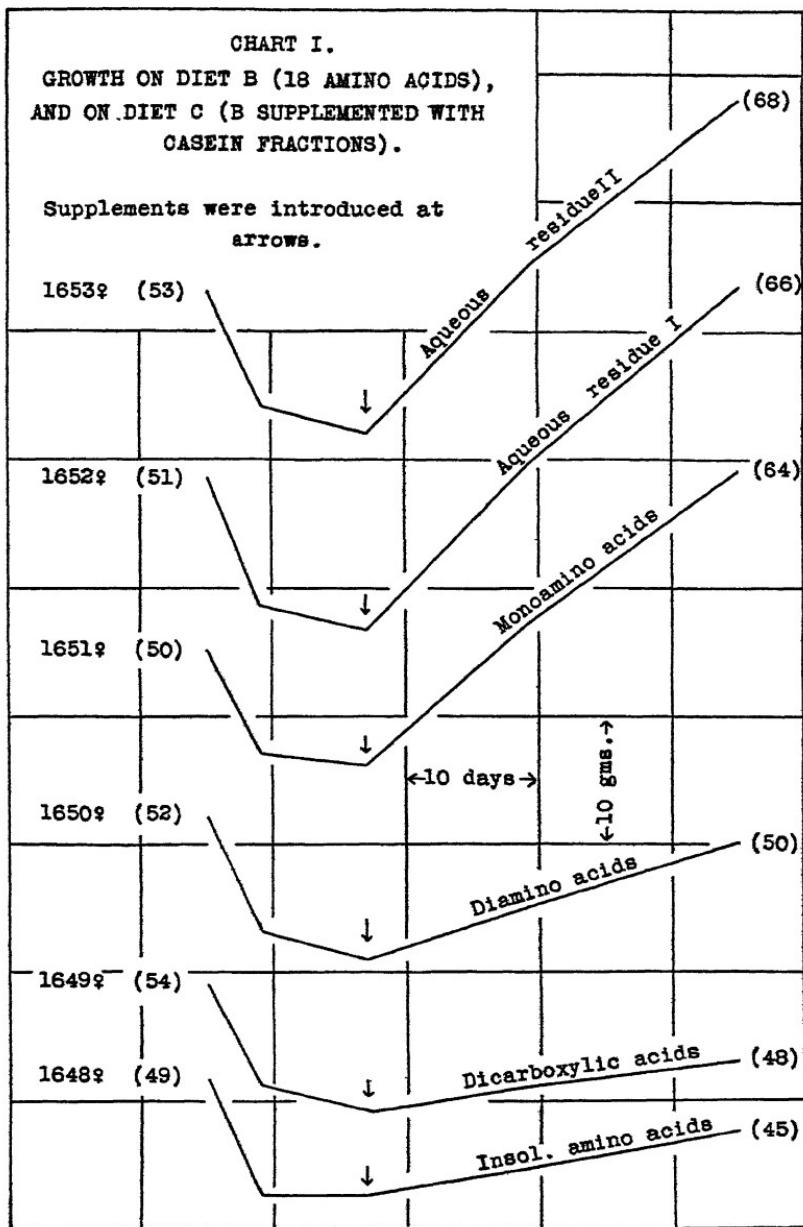
CHART I. The numbers in parentheses denote the initial and final weights of the rats.

CHART II.
GROWTH ON DIET B (18 AMINO ACIDS),
AND ON DIET C (B SUPPLEMENTED WITH
CASEIN FRACTIONS).
Supplements were introduced at
arrows.

1658♀ (49)

Aqueous residue II   (63)

1657♀ — (52)

Monoamino acids   (65)

1656♀ (55)

Diamino acids   (53)

1655♀ (52)

Dicarboxylic acids   (48)

←10 days→

←10 gms.→

1654♀ (54)

Insol. amino acids   (45)
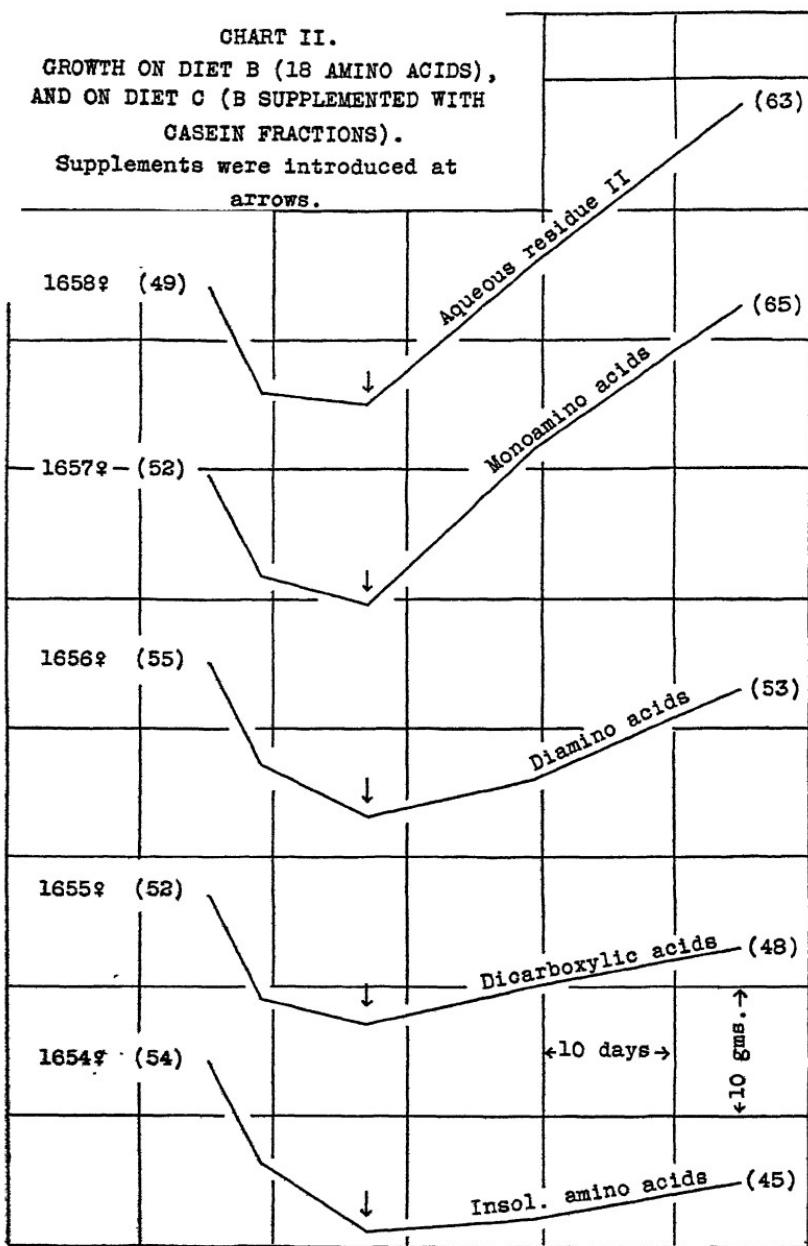
CHART II. The numbers in parentheses denote the initial and final

it is evident that the fractionation resulted in a concentration of the active material in the monoamino acid group, and in the aqueous residues. Fractions 1, 2, and 3 containing the insoluble, dicarboxylic, and diamino acids, respectively, exerted only slightly beneficial effects. Probably this moderate improvement in the quality of the food is to be attributed to a contamination of the fractions in question with traces of the growth essential present in the monoamino acids and aqueous residues. The behavior of

CHART III.
GROWTH ON DIET B (18 AMINO ACIDS), AND ON
DIET C (B SUPPLEMENTED WITH THE PROLINE FRACTION).
Supplements were introduced at arrows.
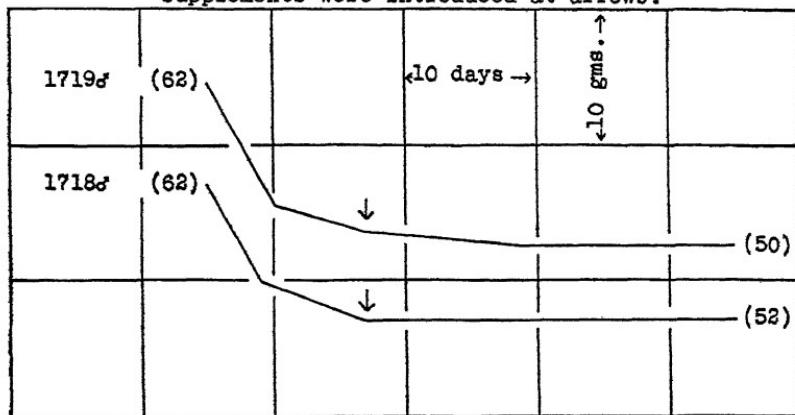
CHART III. The numbers in parentheses denote the initial and final weights of the rats.

the animals which received the dicarboxylic acids provides conclusive proof of the fact that the absence of hydroxyglutamic acid was not the limiting factor in our amino acid mixtures. The experiments in which the crude proline fraction was employed as the supplement (Chart III) show that this material was devoid of activity.

In view of the finding that the growth essential was present in approximately equal proportions in the monoamino acid fraction and in the two aqueous residues, it seemed probable that further

extraction of the aqueous residues with butyl alcohol might lead to the recovery of additional active material.   Furthermore, if

TABLE II

Total Changes in Body Weight and Total Food Intakes on Diet C Containing 5 Per Cent of Casein Fractions

The experiments covered 28 days each.

Rat No and sex	Litter	Casein fraction	Total change in weight	Total food intake
			gm.	*gm.*
1648 ♀	A	Less soluble amino acids	+5	64
1654 ♀	B	(separated by direct	+4	78
1659 ♂	C	crystallization)	+6	67
1685 ♂	D		+1	104*
1649 ♀	A	Dicarboxylic amino acids	+4	72
1655 ♀	B	(precipitated by ba-	+6	92
1660 ♂	C	rium hydroxide and	+4	81
1686 ♂	D	alcohol)	+4	92
1650 ♀	A	Diamino acids (precipi-	+9	69
1656 ♀	B	tated by phosphotung-	+10	84
1661 ♂	C	stic acid)	+6	87
1687 ♂	D		+6	113*
1651 ♀	A	Monoamino acids (ma-	+23	103
1657 ♀	B	terial removed by two	+23	111
1662 ♂	C	butyl alcohol extrac-	+22	128*
1688 ♂	D	tions)	+22	128*
1652 ♀	A	First aqueous residue	+27	95
1689 ♂	D	(less soluble acids)	+34	130*
1653 ♀	A	Second aqueous residue	.+26	103
1658 ♀	B	(more soluble acids)	+23	93
1663 ♂	C		+20	92
1690 ♂	D		+27	133
1718 ♂	E	Proline fraction	0	117*
1719 ♂	E		−1	104*

* This animal scattered food rather badly. This probably accounts for the high figure representing the food intake.

the dietary essential is removed only slowly with the organic solvent, as appeared to be the case, one might expect to secure a fraction of greater potency after most of the more readily soluble

CHART IV.
GROWTH ON DIET B (18 AMINO ACIDS),
AND ON DIET C (B SUPPLEMENTED WITH THE
REFRACTIONATED AQUEOUS RESIDUES OF
CHARTS I AND II.

Supplements were introduced at arrows.
Figures above arrows represent the
percentages of the supplementing
material.
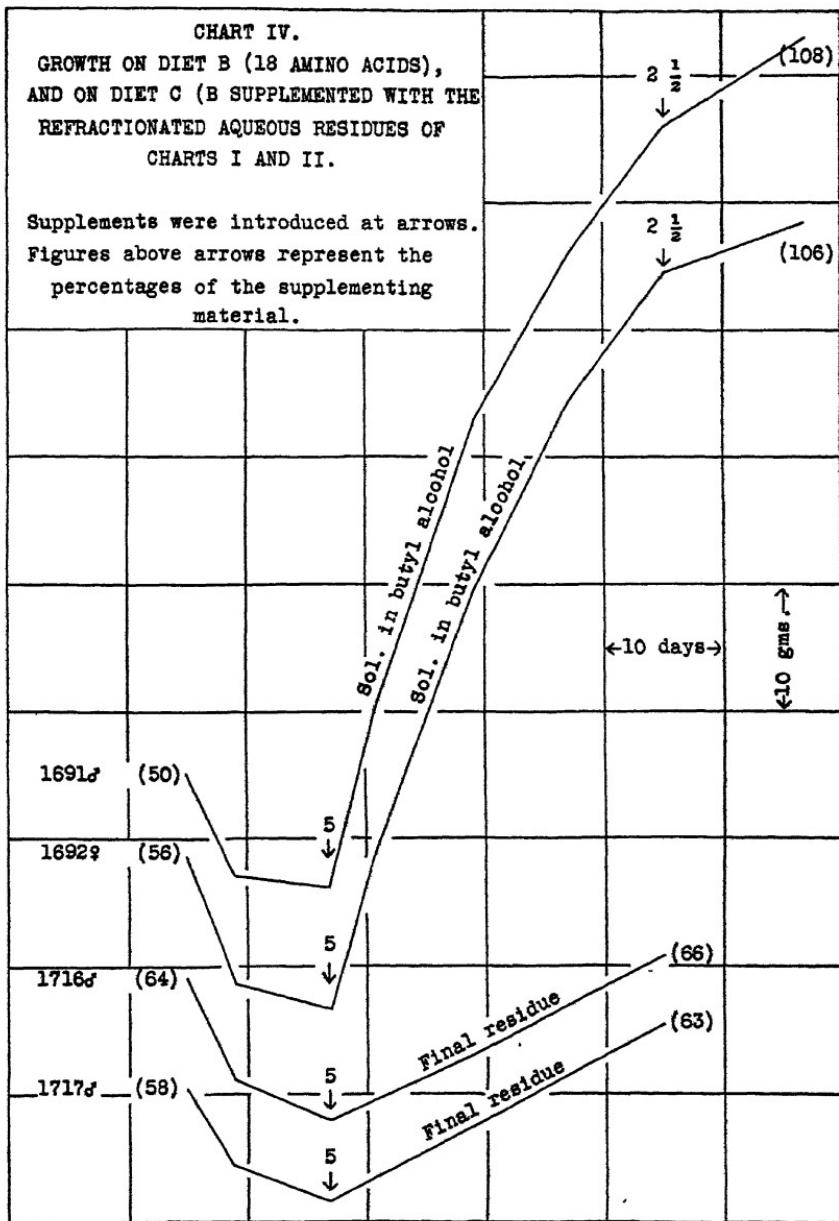
CHART IV. The numbers in parentheses denote the initial and final weights of the rats.

amino acids had been removed.   Proceeding upon this principle, therefore, we undertook an additional fractionation of the combined aqueous residues.   For this purpose 70 gm. of the latter were dissolved in 500 cc. of distilled water, and extracted five times with 1 liter portions of butyl alcohol.   The combined extracts were then evaporated to dryness under diminished pressure, yielding 21 gm. of material.   This and the final aqueous residue, left after evaporation of the water layer, were tested physiologically as in the preceding experiments.   The results were most striking.   They are summarized in Chart IV and in Table III.   It

TABLE III

*Further Concentration of the Active Material Present in the Aqueous Residues.
Total Changes in Body Weight and Total Food Intakes on Diet C
Containing 5 Per Cent of (a) the Material Removed by Five Additional Butyl Alcohol Extractions, and (b) the Final Residue*

The experiments covered 28 days each.*

Rat No. and sex	Litter	Casein fraction	Total change in weight	Total food intake
			gm.	*gm.*
1691♂	D	Material removed by	+60	141
1692♀	D	butyl alcohol	+58	165
1716♂	E	Material not removed by butyl alcohol	+13	85
1717♂	E		+14	89

* The experiments upon Rats 1691 and 1692 were continued for 40 days as shown in Chart IV, but for purposes of comparison with the other data in this and Table II the figures here recorded are for the first 28 days only.

will be observed (Rats 1691 and 1692) that *with 5 per cent of the butyl alcohol-soluble material in the diet normal growth was attained.* After 28 days, the proportion of the supplement was decreased to 2.5 per cent.   Even then growth continued, but at a diminished rate.   On the other hand, the final water residue (Rats 1716 and 1717) showed a marked decrease in activity though still permitting moderate growth.

The figures in Tables II and III representing food intakes are of interest in demonstrating the relationship of appetite and dietary adequacy.   As is noted in Table II, some of the animals scattered the food rather badly thus giving figures which are cer-

tainly too high. But these being omitted from consideration, it will be seen that in general the better the nutritive quality of the food, as indicated by the growth rate, the greater is the food consumption. Unquestionably, rats will consume adequate amounts of diets containing synthetic mixtures of amino acids when all necessary components are present in the food. This aspect of the problem need not be further emphasized at this time.

The fractionation outlined above has been repeated three times in this laboratory on three different lots of casein, and consistent results have been secured in each case. Furthermore, we have fractionated casein by an entirely different procedure (unpublished data), and have again succeeded in concentrating the growth essential in the monoamino acid group. Under the circumstances we believe that our data afford convincing proof for the hypothesis set forth in Paper I of this series, namely, that growth-promoting proteins contain at least one indispensable dietary component other than the generally recognized amino acids.

At present we have no information as to the chemical nature of the material in question although we have learned a great deal concerning its properties. We early suspected that the active compound might be aminobutyric acid (cf. Foreman, 1913, and Abderhalden and Weil, 1913), or one of the amino acids described by Schryver and his associates (1925, 1925–26, 1926, 1927). For reasons which will be considered at a later date, none of these appears to be the growth stimulant present in our active fractions. Identification must, of course, await isolation in pure condition. This we have hopes of being able to accomplish inasmuch as we have at hand, in the remarkable growth-stimulating action of the substance, a biological test which enables us to follow it with ease. We are now preparing large quantities of the active fraction, and are proceeding along three different lines to effect its further purification. If we are successful, it will then be possible to determine with certainty which of the known amino acids are essential for life.

SUMMARY

The amino acids of hydrolyzed casein have been subjected to fractionation into several groups each of which has been tested as to its supplementing effect upon a diet containing a synthetic

mixture of amino acids. The results indicate that the mono-amino acid fraction (butyl alcohol-soluble portion) contains a material which exerts a remarkably stimulating effect upon the growth of rats. The data are believed to provide convincing proof for the presence in casein of a hitherto unknown dietary essential.

BIBLIOGRAPHY

Abderhalden, E., and Weil, A., *Z. physiol. Chem.*, **88**, 272 (1913).

Dakin, H. D., *Biochem. J.*, **12**, 290 (1918).

Foreman, F. W., *Biochem. Z.*, **56**, 1 (1913).

Kingston, H. L., and Schryver, S. B., *Biochem. J.*, **18**, 1070 (1924).

Osborne, T. B., Leavenworth, C. S., and Nolan, L. S., *J. Biol. Chem.*, **61**, 309 (1924).

Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

Schryver, S. B., and Buston, H. W., *Proc. Roy. Soc. London, Series B*, **99**, 476 (1925–26); **100**, 360 (1926); **101**, 519 (1927).

Schryver, S. B., Buston, H. W., and Mukherjee, D. H., *Proc. Roy. Soc. London, Series B*, **98**, 58 (1925).

CAROTENASE. THE TRANSFORMATION OF CAROTENE TO VITAMIN A IN VITRO*

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The work of Moore, Capper, and Drummond and their co-workers (3–5, 11) has definitely established the rôle of carotene as a precursor of vitamin A. When carotene, or substances containing it, were fed to vitamin A-free rats or fowls, the livers of such animals were found to contain vitamin A. Moore (12) showed that the liver contains more vitamin A than other tissues, and concluded that the conversion takes place in that organ. If an enzyme, carotenase, is responsible for this transformation, it should be possible to prepare vitamin A from carotene *in vitro* by incubation with whole liver or liver extracts. It is the purpose of this paper to outline a series of experiments which demonstrate that such a reaction does take place.

EXPERIMENTAL

In the present study the presence of vitamin A was detected by ultra-violet absorption spectra methods, since vitamin A is characterized by a broad absorption band with a maximum near $328m\mu$ (2, 6, 8, 13, 14, 18). A Hilger E-2 quartz spectrograph in conjunction with a sector photometer and a tungsten-steel spark was used for the photographs.

The absorption curves of carotene, achroocarotene (16), and carotene decolorized by oxidation are presented in Fig. 1. These curves agree with those of Duliere, Morton, and Drummond (7), Capper (2), and McNicholas (10) for carotene and oxidized carotene. The absence of a band at $328m\mu$ indicates that decoloriza-

* An abstract of this work is in press in *Science* (1931).

tion of carotene by heat or oxidation does not yield vitamin A, an observation which has been confirmed by biological assays. It should be noted that carotene has bands at $280m\mu$ and $345m\mu$, and no band at $328m\mu$, and that its absorption is greater than either of its derivatives as indicated by the concentration used in the cell.
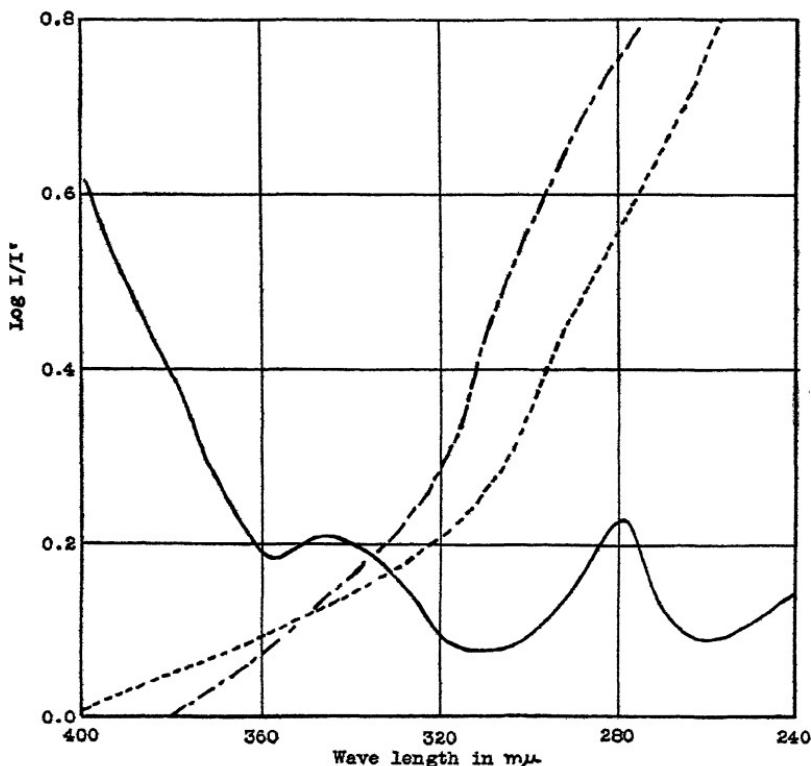
FIG. 1.————absorption spectrum of carotene in chloroform, 1:75,000; ———— achroocarotene in chloroform, 1:50,000; — — — - oxidized caro-tene in chloroform, 1:10,000.

Fresh whole liver from vitamin A-free rats was used in the first attempts to produce vitamin A *in vitro*. The rats had been fed on a diet consisting of sucrose (46 per cent), lard (24 per cent), extracted casein (18 per cent), dried yeast[1] (8 per cent), salts[2] (4

[1] Courtesy of the Northwestern Yeast Company.
[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **32**, 317 (1917).

per cent), and viosterol[3] (10 drops per kilo of diet).   When a rat became deficient in vitamin A, as denoted by a rapid loss of weight, it was killed by a blow on the head, and its liver extirpated.

The liver from Rat 347 (Fig. 2) weighed 5 gm.   It was thoroughly ground with sand, 15 cc. of a phosphate buffer solution at pH 7.45, and approximately 2 cc. of ethyl laurate in which had been dissolved 2 mg. of carotene (from lettuce (15)).   The mix-
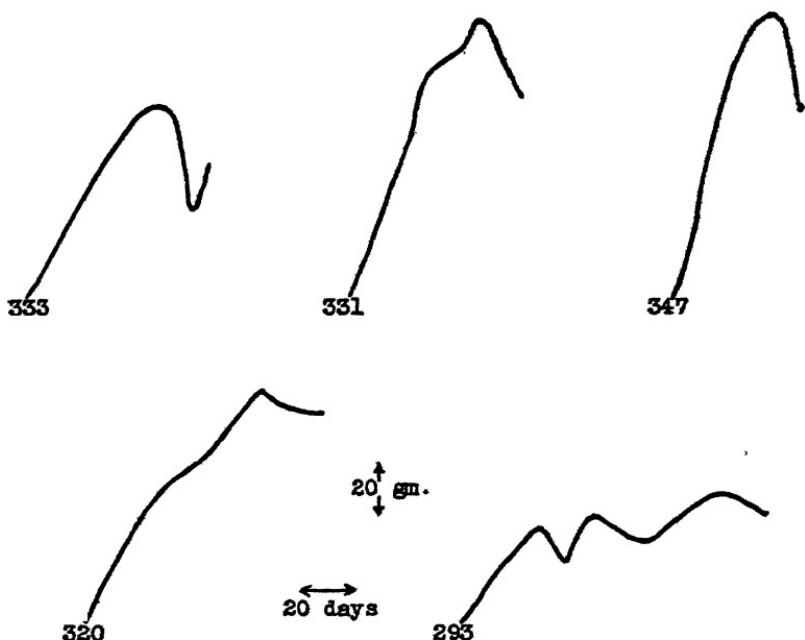
Fig. 2. Growth curves of rats on vitamin A-free diet. Rat 333 was fed 0.010 mg. of carotene per day for 8 days before it was killed.

ture was allowed to stand 24 hours at 38°, 10 cc. of 10 per cent potassium hydroxide were added, and the incubation continued for another 24 hours.   The saponified material was then thoroughly extracted with ether, the ether was washed, dried over anhydrous sodium sulfate, and evaporated to dryness.   The residue was dissolved in approximately 300 times its weight of chloroform, and the absorption spectrum photographed.   A

[3] Courtesy of Mead Johnson and Company.

distinct band at $328m\mu$ (Fig. 3) indicated that vitamin A was
present.   Although the ether extract had been slightly yellow,
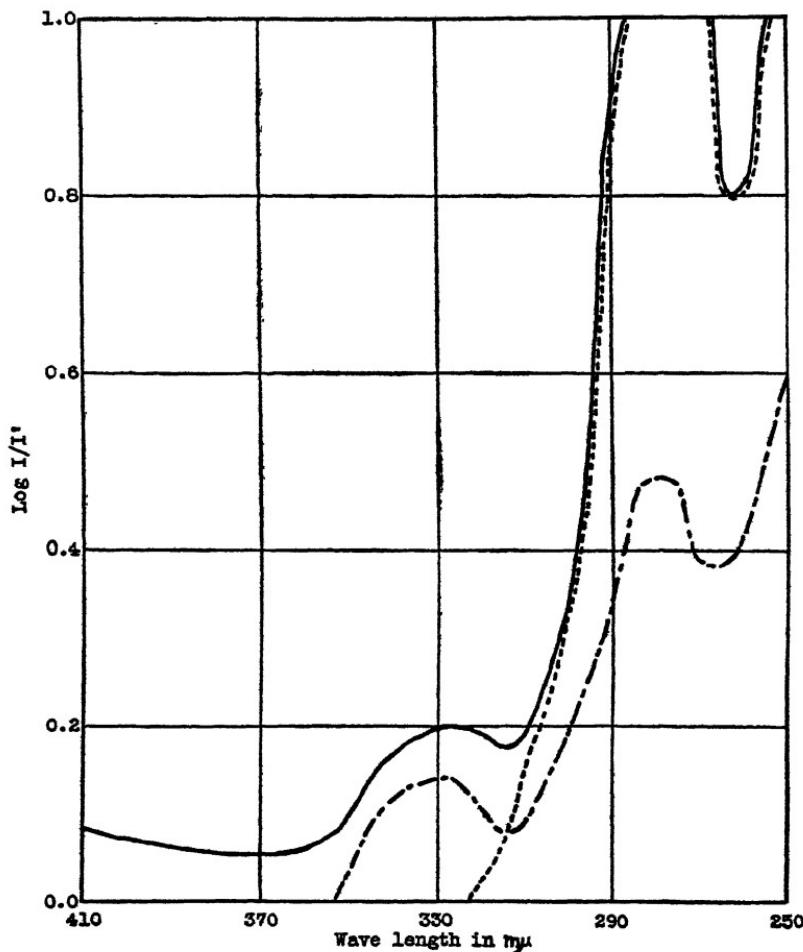
Fig. 3.   —— absorption spectrum of unsaponifiable liver lipids of
Rat 347, carotene added, in chloroform, 1:300; ---------- same of Rat 331,
control, in chloroform, 1:300; ———- same of Rat 333, previously fed
carotene, in chloroform, 1:500.

there was not sufficient carotene remaining to give the typical
carotene bands.   The same·procedure was followed in two more
experiments with similar results.

The liver from Rat 331 (Fig. 2) was treated in the same way except that the ethyl laurate added contained no carotene. The absorption spectrum (Fig. 3) showed no indication of a band at $328m\mu$, nor did the extracts from two more livers treated similarly absorb in this region. Capper (3) was likewise unable to distinguish a band at $328m\mu$ in the absorption spectra of liver oils from vitamin A-depleted rats.

Two rats were allowed to become depleted of vitamin A stores; they were then fed carotene dissolved in ethyl laurate, and when growth had become rapid, they were killed and their livers examined for vitamin A. The spectra of both oils were similar; to save space, as in the previous cases, the protocol of only one is included. The liver of Rat 333 (Fig. 2) was cut into small pieces and dissolved in 10 per cent potassium hydroxide by warming for 24 hours. The saponified mixture was extracted as in the previous cases, and the absorption of the extracted material determined. The band at $328m\mu$ (Fig. 3) was again evident, confirming the observations of Moore, Capper, and others (3, 5, 11) that carotene was changed in the body to vitamin A, and from the similarity of the absorption curves, justifying the assumption that the band found after the *in vitro* incubation of carotene with liver tissue was due to vitamin A.

An active extract of carotenase was prepared in the following manner: The livers from Rats 293 and 320 (Fig. 2) were combined, thoroughly ground with sand and 50 cc. of toluene-water, and the mixture allowed to incubate for 24 hours at 37°. The digest was then filtered through cheese-cloth and coarse filter paper, and the filtrate reserved as a solution of the enzyme. 2 cc. of the liver extract were thoroughly extracted with ether. The ether was evaporated to dryness, the residue dissolved in a small amount of chloroform, and its absorption spectrum determined as before (Fig. 4). The absence of a band at $328m\mu$ indicated that the extract contained no appreciable amounts of vitamin A.

A colloidal solution of carotene in water was prepared by the method suggested by Fodor and Schoenfeld (9). A stable colloid, exhibiting a marked Tyndall effect, was obtained which contained approximately 0.02 mg. of carotene per cc. 6 cc. of this solution were mixed with 2 cc. of the liver extract, and allowed to incubate for 36 hours. The ether extract was colorless, indicating that the

carotene had been changed during the incubation.   An absorption spectrum of the residue in chloroform showed a band at $328m\mu$ (Fig. 4).

2 cc. of the liver extract were heated to boiling, cooled, the solution of carotene added, and the incubation allowed to proceed
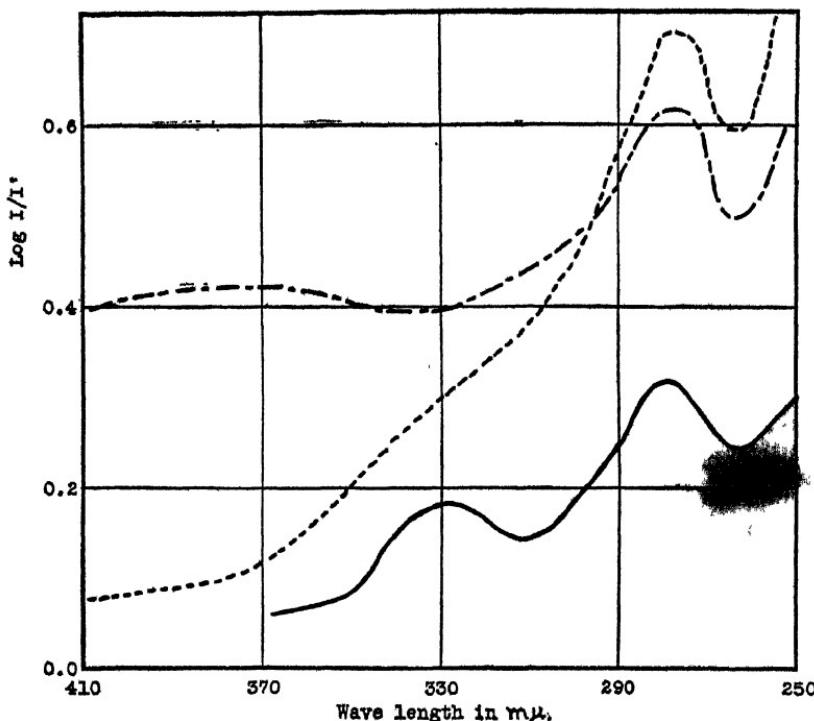
FIG. 4. ---------- absorption spectrum of ether-soluble constituents of liver extract, in chloroform; ——— ether-soluble constituents of liver extract after incubation with carotene in chloroform; — — —— ether-soluble constituents of heated liver extract after incubation with carotene, in chloroform.

exactly as before.   The ether extract after incubation was yellow, but the color faded during the separate manipulations, and the concentration of the remaining carotene was not sufficient to give the typical carotene spectrum.   The absence of the band at $328m\mu$ (Fig. 4) demonstrated the thermolability of the agent responsible for the reaction.

Von Euler and his coworkers (8) have recently shown that dihydrocarotene retains some of the growth-promoting property of carotene; further, that a mixture of the more fully hydrogenated products has three absorption bands in the ultra-violet region, one of which has a maximum at $328m\mu$. Their results suggest that vitamin A may be related to reduced carotene, from which it follows that the function of carotenase might be that of catalyzing a particular type of reduction. Bruins, Overhoff, and Wolff (1) determined the molecular weight of vitamin A by a comparison of the diffusion constants of carotene and the vitamin. From their results, they deduce a molecular weight of 330 (carotene, mol. wt., 536) and state that "the value obtained causes the assumption of a simple chemical relation between vitamin A and carotene to appear improbable." It is possible that vitamin A is a reduced fragment of the carotene molecule, and that reduction of carotene itself creates that part of the molecule responsible for the absorption at $328m\mu$.

Previous workers have noted a band at $280m\mu$ in the absorption spectra of cod liver oils (13, 17), and Capper (3) called attention to inflections in that region in the absorption spectra of rat liver oils. The bands were in no case as pronounced as those observed in the present study. Both carotene (Fig. 1) and its isomer, lycopin (unpublished observations), have a band at $280m\mu$, although lycopin differs from carotene in lacking the band at $345m\mu$. One of the three important bands of ergosterol is at 280 $m\mu$. Although it may be a chance occurrence, it is possible that some molecular configuration, common to carotene, lycopin, ergosterol, and the unknown substances in fish and animal oils, is responsible for the absorption at $280m\mu$. The character of the compounds present in liver which contain the configuration absorbing at $280m\mu$ remains to be determined. Whatever compound is responsible, it seems to be present in equal amounts in the separate rat livers (Fig. 3).

The concentration of the solutions used to determine the absorption curves presented in Fig. 4 was not known since the amount of material used was too small to be weighed; consequently the heights of the bands are not proportional to the total amount of absorbing substance (a condition which would be true if the

same concentration of the separate oils had been used). A more exact comparison of the vitamin A content can be obtained by superimposing the peaks of the $280m\mu$ bands on one another, that is, by assuming that each 2 cc. of liver extract contained an equal amount of the unknown substance absorbing at $280m\mu$. If such a comparison is made with the curves in Fig. 3, it will be seen that the liver treated with carotene *in vitro* contained considerably less vitamin than that of the rat previously fed carotene.

Further study of the absorption curves in Figs. 3 and 4 reveals that, by using a liver extract, it has been possible to reduce the relative amount of the unknown substance. The control curve of the liver extract falls off less sharply than the control curve of the whole liver, and the vitamin A content is proportionately several times greater in the experiments with liver extract.

SUMMARY

Carotene can be changed to vitamin A by incubation with fresh liver tissue or with an aqueous extract of liver. The agent responsible for the transformation appears to be an enzyme, provisionally called carotenase, since it is destroyed by heat. The conversion of carotene to vitamin A *in vivo* and the usefulness of ultra-violet absorption spectrum methods for the detection of vitamin A are confirmed.

BIBLIOGRAPHY

1. Bruins, H. R., Overhoff, J., and Wolff, L. K., *Biochem. J.*, **25**, 430 (1931).
2. Capper, N. S., *Biochem. J.*, **24**, 453 (1930).
3. Capper, N. S., *Biochem. J.*, **24**, 980 (1930).
4. Capper, N. S., McKibbin, I. M. W., and Prentice, J. H., *Biochem. J.*, **25**, 265 (1931).
5. Drummond, J. C., Ahmad, B., and Morton, R. A., *J. Soc. Chem. Ind.*, **49**, 291 T (1930).
6. Drummond, J. C., and Morton, R. A., *Biochem. J.*, **23**, 785 (1929).
7. Duliere, W., Morton, R. A., and Drummond, J. C., *J. Soc. Chem. Ind.*, **48**, 316 T (1930).
8. von Euler, H., Karrer, P., Hellström, H., and Rydbom, M., *Helv. Chim. Acta*, **14**, 839 (1931).
9. Fodor, A., and Schoenfeld, M., *Biochem. Z.*, **233**, 243 (1931).

10. McNicholas, H. J., *Bur. Standards J. Res.*, **7**, 171 (1931).
11. Moore, T., *Biochem. J.*, **24**, 692 (1930).
12. Moore, T., *Biochem. J.*, **25**, 275 (1931).
13. Morton, R. A., and Heilbron, I. M., *Biochem. J.*, **22**, 987 (1928).
14. Morton, R. A., Heilbron, I. M., and Thompson, A., *Biochem. J.*, **25**, 20 (1931).
15. Olcott, H. S., and Mattill, H. A., *J. Biol. Chem.*, **93**, 59 (1931).
16. Olcovich, H. S., and Mattill, H. A., *J. Biol. Chem.*, **91**, 105 (1931).
17. Schlutz, F. W., and Morse, M., *Am. J. Dis. Child.*, **30**, 199 (1925).
18. Takahashi, K., Nakamiya, Z., Kawakami, K., and Kitasato, T., *Sc. Papers Inst. Physic. and Chem. Research*, Tokyo, **3**, 81 (1925).

STUDIES ON THE CHEMICAL COMPOSITION OF THE HUMAN SKELETON

I. CALCIFICATION OF THE TIBIA OF THE NORMAL NEW BORN INFANT*

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The deposition of inorganic elements in the human fetus and new born infant has been studied principally by one of two methods, namely, by means of analyses of the entire bodies of the fetuses and new-born or by means of mineral balance experiments on pregnant women.

Numerous reports of the inorganic composition of the human fetus and new born infant include those of Fehling (1), Giacosa (2), de Lange (3), Michel (4), Hugounenq (5), Camerer and Söldner (6), and more recently of Givens and Macy (7) and of Schmitz (8). It is not evident from these reports whether in all cases these analyses can be credited with being those of essentially normal fetuses and new-born since events associated with their deaths have usually been omitted from the reports. The total amounts as well as the percentages of ash or inorganic elements based on the fresh or dry weights of new born infants have shown considerable variability; thus Fehling found 58.08 gm. of total ash or 3.3 per cent of ash in the body of a new born infant, Michel found 112.49 gm. of total ash or 3.37 per cent of ash in the body of a new born infant, Camerer found as an average of six new born infants 2.70 per cent of ash, the individual values varying from 53.89 to 87.05 gm. and from 2.06 to 3.10 per cent of ash, and Hugounenq reports 96.76 and 106.16 gm. of ash or 3.55 and

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3.52 per cent of ash respectively in two full term fetuses analyzed by him.   It seems to be quite well established, therefore, that the bodies of new born infants vary considerably with respect to their content of inorganic elements, but whether this is due to differences in degree of calcification of the skeleton, to differences in the ratio of skeleton to soft tissue, or both is not so apparent.

The interpretation of mineral balance experiments on pregnant women is complicated by the fact that it never has been possible to ascertain with any greater precision than a rough approximation the actual amounts of the inorganic elements deposited in the fetus during the particular interval when the metabolic balance studies were in progress.   Neither have mineral balance experiments been studied throughout the entire period of gestation. Recently, however, Coons and Blunt (9) and Macy *et al.* (10) have reported mineral balance studies on pregnant women at frequent intervals during gestation.   The former of these compared the average daily calcium retention at definite intervals with the assumed average daily fetal demand for calcium as calculated from the published data of fetal analyses by Schmitz (8) and by Hugounenq (5).   It was found that the daily calcium retention of the three subjects with greatest positive calcium balances corresponded almost exactly with the supposed fetal demand and all but one of the six other subjects retained calcium far inadequate to meet the assumed fetal requirements.   The indications were, as these authors point out, that most of these women were either depositing only small amounts of calcium in the fetus or were drawing upon their own skeletons to meet the calcium need of the growing fetus.

Macy *et al.* (10) found, as a result of their mineral balance experiments on pregnant women during the last half of pregnancy, that appreciable negative calcium balances frequently existed in their subjects and that large variations in the calcium balances were usual.

Very excellent reviews of the literature dealing with the fetal mineral requirement in relation to the metabolism of pregnant women have been published by Hoffström (11), Murlin (12), Shohl (13), Coons and Blunt (9), and by Macy *et al.* (10).

The formation of the osseous component of bone in embryonal vertebrates implies a transformation of embryonal connective tis-

sue into bone by specially modified connective tissue cells, the osteoblasts. Bone, therefore, is an extremely rigid form of connective tissue, the osseous component of which consists of a hyaline trabeculated organic matrix of definite structure in which are deposited variable quantities of calcium salts. The approximately constant chemical composition of the inorganic material of bone has been mentioned in some of the earliest records of bone analyses. Hoppe-Seyler observed that the ratio of calcium to phosphorus in bone ash was about the same as in apatite. This ratio of calcium to phosphorus has been further noted to be constant although the bone may be gaining or losing its inorganic constituents. A very recent comparative study of bone and various calcium salts by means of x-ray spectrograms by Roseberry et al. (14) confirms the conclusions of several other earlier workers that bone contains a crystalline mineral of the apatite series, having the type formula, $nCa_3(PO_4)_2 \cdot CaCO_3$ or $nCa_3(PO_4)_2 \cdot CaX_2$ where n is not greater than 3 nor less than 2.

The chemical processes associated with the deposition of this mineral in bone are little understood. It is, of course, necessary that the calcium salts be present in suitable quantities in the fluid bathing the osteoblasts, which in turn necessitates certain equilibrium concentrations of calcium, phosphate, and carbonate in the circulating blood and lymph. Robison (15) has shown that in the carriage of calcium salts to the bone, a part may be played by a hexose monophosphoric ester which has been detected in blood and which forms soluble salts with calcium. Under the action of an enzyme present in ossifying cartilage this calcium salt is hydrolyzed, and calcium phosphate with a certain amount of carbonate is precipitated in the organic matrix around the osteoblasts.

It has been amply demonstrated also that vitamin D plays an important part in regulating the deposition of the apatite mineral in ossifying cartilage.

The work of Aron and Sebauer (16), Sherman and MacLeod (17), and of Sherman and Booher (18) has shown that the calcium content of the skeleton of animals is in large measure also dependent upon the calcium intake. However, in the case of the new born infant where the young organism has been parasitic upon the maternal organism, the influence of the maternal diet upon the

calcification of the skeleton of the infant, so far as we have been able to learn, has not been investigated.

In addition to the factors enumerated above, calcification of the skeleton is in some manner dependent upon the secretions of certain of the endocrine glands, notably of the pituitary and parathyroid glands. Bone, furthermore, is not a fixed tissue but an active ever-changing living tissue, the formation of which is regulated by a complexity of interrelated factors normally acting with very precise coordination.

The purpose of the present investigation was to study the degree of, and relative variability in, the calcification of the skeleton of the normal new born infant, the tibia being selected as an index of skeletal calcification because of expediency in handling and because of the striking involvement of the tibia in early stages of rickets and, therefore, sensitiveness as a measure of the variability in the degree of calcification in young growing bone.

Procedure

The excision and analyses of the tibias of the new born infants, here reported, were preceded by a thorough postmortem examination of the infant by one of us (G.H.H.). Records of the periodic antepartum examinations of the mothers were freely consulted and the information obtained therefrom in conjunction with the postmortem findings with respect to the new born infant constituted the basis for accepting or rejecting the infant for further study. In the present investigation only the tibias of those new born infants, which were regarded as truly normal full term fetuses when judged by the above criteria, were excised and analyzed and in no case were any results rejected after the selection of the infant had been made.

Both right and left tibias of the new born infant were excised as soon as feasible after the death of the infant and freed from adhering soft tissue, extreme care being taken to keep the tibias completely intact and unabraded. The approximate weights of the fresh tibias have been recorded in one of our tables and are subject to the inconstant errors incurred in the time-consuming operation of quantitative removal of the adhering soft tissue. The net tibias were dried for some hours at 100°, crushed between several folds of ashless quantitative filter paper, extracted for 30 hours

with 95 per cent alcohol and then for 15 hours with ether in Soxhlet apparatus which siphoned at intervals of about 30 minutes, transferred to weighed silica dishes, and quickly dried to constant weight in an electric oven at 100°. The dry extracted tibias were carefully charred and finally reduced to a gray-white ash in a muffle furnace regulated at dull red heat, the ashing process being continued until the weight of the ash was constant. The ash of each tibia was dissolved in dilute hydrochloric acid and diluted to a convenient volume, aliquots of which were used for the quantitative determination of calcium according to the method of McCrudden.

In order that differences in calcium or vitamin D intake by the mothers might possibly be of use in the interpretation of the results, a record of the principal sources and relative quantities of these two food factors in the maternal diet was personally solicited in each case.

In Table I are recorded certain physical measurements of the entire bodies and of the tibias of the four male and four female new born infants, together with a very brief indication of any notable incident associated with the death of the infant. In each case the occurrence of death may be regarded as due either to an accident attendant to birth or to a condition arising so *suddenly* in the maternal organism at the end of a normal gestation period that the development of the fetus could not conceivably have been disturbed.

In Table II are recorded the results of the analyses of the right and left tibias of each of the eight normal new born infants. The total amount of ash and of calcium in each of the eight pairs of tibias varies in the same order as the dry extracted weights of the tibias and in almost exactly the same proportions. The average percentage of ash in the sixteen tibias of the eight new born infants on the basis of the dry extracted weights of the tibias was 44.38 per cent with a coefficient of variation of 1.7 per cent; the average percentage of calcium in these same tibias was 16.62 per cent with a coefficient of variation of 2.2 per cent. The average percentages of ash and of calcium differed slightly when calculated separately for males and females as shown in Table II,

but this difference is apparently insignificant since the range of individual values is quite the same for males and females. It is evident from these results that the degree of calcification of the tibias of normal new born infants is almost invariant as shown by the very small values of the coefficients of variation for the

TABLE I

Description of Normal New Born Infants

Autopsy No.	Sex	History	Weight	Length	Length of tibias	Antero-posterior diameter of tibia shaft	Approximate fresh weight of tibias
			gm.	*cm.*	*cm.*	*cm.*	*gm.*
A-31-12	M.	Birth trauma (lived 20 min.)	4615	57	7.9	0.75	15.1
					7.9	0.70	15.1
A-31-51	"	Strangulation (umbilical cord twisted about neck)	3600	54	7.9	0.70	11.1
					7.9	0.65	11.3
A-31-70	"	Hemorrhage from lacerations (lived 12½ hrs.)	3154	51	7.9	0.69	13.2
					7.9	0.73	13.2
A-31-62	"	Cerebral trauma (lived 4 min.)	3450	53	7.9	0.70	11.9
					8.0	0.73	12.7
A-31-53	F.	Eclampsia; sudden termination of gestation; normal fetus	3945	55	8.0	0.69	13.0
					8.0	0.70	12.9
A-31-68	"	Cerebral hemorrhage (lived 20 min.)	3640	53	7.7	0.63	10.4
					7.8	0.61	10.2
A-31-73	"	Found dead in crib (lived about 10 hrs.)	3252	50	7.8	0.68	11.5
					7.8	0.63	11.6
A-31-74	"	Influenza eclampsia; sudden termination of gestation; normal fetus	3250	54	7.9	0.63	12.0
					7.9	0.60	12.1

percentages of ash and of calcium. The differences between the percentages of ash or of calcium in the two tibias of the same infant are frequently as great as the standard deviation for the entire series of tibias. Considering the complexity of factors involved in the calcification of the osseous component of bone, as briefly summarized above, this practically constant degree of calcification

of the skeleton of the normal new born infant must represent an exceedingly precise coordination of chemical transformations. The numerous reports in earlier literature of the mineral composition of the human fetus at various stages in its development and of the

TABLE II

Analyses of Tibias of Normal New Born Infants

Autopsy No.	Sex	Dry extracted weight of tibias	Ash content of tibias		Ca content of tibias		Ca content of ash
		gm.	*gm.*	*per cent*	*gm.*	*per cent*	*per cent*
A-31-12	M.	5.211	2.296	44.1	0.858	16.5	37.4
		5.207	2.289	44.0	0.858	16.5	37.5
A-31-51	"	4.237	1.877	44.3	0.707	16.7	37.7
		4.276	1.863	43.6	0.701	16.4	37.6
A-31-70	"	4.654	2.131	45.8	0.791	17.0	37.1
		4.662	2.113	45.3	0.787	16.9	37.3
A-31-62	"	4.419	2.015	45.6	0.755	17.1	37.5
		4.510	2.013	44.6	0.756	16.8	37.6
Averages......................				44.66		16.74	37.46
Coefficients of variation.........				1.7		1.4	0.5
A-31-53	F.	4.586	2.095	45.7	0.783	17.1	37.4
		4.580	2.081	45.5	0.783	17.1	37.6
A-31-68	"	3.560	1.566	44.0	0.591	16.6	37.8
		3.655	1.613	44.1	0.607	16.6	37.6
A-31-73	"	4.119	1.797	43.6	0.668	16.2	37.2
		4.115	1.780	43.3	0.663	16.1	37.3
A-31-74	"	3.971	1.718	43.3	0.633	16.0	36.9
		3.999	1.736	43.4	0.641	16.1	36.9
Averages......................				44.11		16.48	37.34
Coefficients of variation.........				2.5		2.5	0.8
Averages of males and females..				44.38		16.62	37.40
Coefficients of variation of males and females...................				1.7		2.2	0.7

new born infant have been valuable contributions in studying the relative rate of gross acquisition of these inorganic elements by the fetus together with indications of the total quantities acquired. That the total amounts of the inorganic elements acquired, by supposedly normal new born infants, have appeared so variable

in the earlier reports has not been due, evidently, to variability in the degree of calcification of the skeletons *but* to variability in the relative sizes of the skeletons. When the inorganic compositions of the new born infants were expressed in terms of percentage composition of the body weight, the results were most

TABLE III

History of the Mothers

Age	Previous pregnancies	Daily milk consumption during gestation	Cod liver oil additions to food during gestation	Autopsy No. of infant
yrs.				
23	Twins 1 yr. previous	1 qt., last 60 days; otherwise confined to cooking uses	Last 60 days	A-31-12
20	None	1 qt., last 45 days; 1 glass previously	Last 45 days	A-31-51
14	None	1 qt., last 72 days. Family of 7 consumed 2 qts. daily previously	Last 72 days	A-31-70
20	One 3 yrs. previous	1 qt., last 4 days; otherwise confined to cooking uses	Last 4 days	A-31-62
40	Three—12, 8, and 5 yrs. previous	1 qt., last 60 days; otherwise confined to cooking uses	Last 33 days	A-31-53
21	Two—one 2 yrs. previous, miscarriage 1 yr. previous	1½ qts., last 14 days. Family of 3 consumed 3 pints daily previously	Last 14 days	A-31-68
17	None	Limited entirely to cooking uses	Last 56 days	A-31-73
30	None	3 glasses throughout, in addition to that used in cooking	Viosterol, last 4 mos.	A-31-74

certainly augmented in their variability due to the fact that the ratio of skeleton to soft tissue varies quite considerably in the new born human infant.

It might be interesting in connection with these results to mention that both Goss and Schmidt (19) and Sherman and Booher

(18) have pointed out the slight variability in the calcium content of the bodies of new born rats analyzed by them. It should be pointed out, however, that at least in the latter case the rats were of the same strain and the variation in weights of the new born rats was negligible.

The pertinent facts in the histories of each of the mothers of the eight normal new born infants studied are given in Table III. The range in the mothers' ages was from 14 to 40 years; the number of previous pregnancies varied from none to four, with one case having given birth to twins just 1 year previous to the birth of the infant included in this study; and the intake of foods rich in calcium, phosphorus, and vitamin D during the gestation period differed widely among the eight mothers. It is apparent from our results that none of these variable factors, within the limits mentioned, were effective in modifying the degree of calcification of the skeleton of the normal new born infant as judged by analyses of the tibias. The normal human fetus is to be regarded, with respect to the acquisition of inorganic elements, as leading an entirely parasitic existence in which the maternal body is sacrificed if need be to the demands of the calcifying fetal skeleton. If the calcium balances of the six maternal subjects with lowest calcium retentions studied by Coons and Blunt (9) represent normal pregnancies, our analyses would indicate that those women were drawing upon the calcium of their own skeletons to meet the calcium demand of the growing fetus rather than that less than the normal degree of calcification of the fetal skeleton was taking place.

In the cases of the youngest mothers, whose own skeletons could not have been calcified to the degree of that of a fully developed adult, this calcium drain in favor of the fetus would seem especially serious. With regard to the seeming lack of influence of relative vitamin D intakes by the mothers in controlling the degree of calcification of the infant skeleton, it might be stated that these mothers probably received adequate amounts of vitamin D from foods other than the cod liver oil additions and as a result of irradiation in sunlight, for most of the mothers consumed vegetables and butter in relative abundance and lived considerably in the out-of-doors. A liberal supply of vitamin D in the maternal diet may be effective in increasing the utilization of the ingested cal-

cium and phosphorus and thus in decreasing the drain on the maternal body, or it may be, as several recent investigations have indicated, that vitamin D has less influence upon the calcium balance than has generally been supposed since the discovery of the antirachitic potency of this vitamin.

The average percentage of calcium in the ash of the sixteen tibias was 37.40 per cent with a coefficient of variation of 0.7 per cent. The range of individual values was from 37.8 to 36.9 per cent. The calcium percentages for the ash of any two tibias of the same infant were never different by more than 0.2 per cent and therefore probably not more different than would be within the limits of experimental error. The percentages of calcium in $3Ca_3(PO_4)_2CaCO_3$ and in $2Ca_3(PO_4)_2CaCO_3'$ are 38.86 and 38.92 per cent respectively. The ash of an entire bone includes not only the inorganic salt deposited in the organic matrix but also the ash of the constituents of the bone marrow and matrix which are richer in phosphorus than in calcium. In view of these facts an average value of 37.40 per cent of calcium with a coefficient of variation of 0.7 per cent is in all probability consistent with the finding that bones contain a crystalline mineral of the apatite series.

SUMMARY

Quantitative determinations of the total ash and calcium content of the tibias of eight normal new born infants, together with a study of the antepartum histories of the mothers support the following conclusions:

1. While the total amounts of ash and of calcium in the tibias varied considerably, the percentages of ash and of calcium in the dry fat-free tibias of normal male and female new born infants were almost invariant.

2. The average percentage of ash in the tibias of the normal new born infants was 44.38 with a coefficient of variation of 1.7 per cent. The average percentage of calcium in these same tibias was 16.62 with a coefficient of variation of 2.2 per cent.

3. With respect to the deposition of the inorganic constituents of its bones, the normal human fetus may be regarded as entirely parasitic upon the maternal organism, since large differences in calcium and phosphorus intake by the mothers did not affect the

degree of calcification of the tibias of the normal new born infants. When the age of the younger mothers was such that active calcification of their own bones should have been taking place simultaneously, this had no appreciable effect on the calcification of the skeletons of the full term fetuses borne by them.

BIBLIOGRAPHY

1. Fehling, H., *Arch. Gynäk.*, **11**, 523 (1877).
2. Giacosa, G., cited by Forbes, E. B., and Keith, H. M., *Ohio Agric. Exp. Station, Bull. 5* (1914).
3. de Lange, C., cited by Forbes, E. B., and Keith, H. M., *Ohio Agric. Exp. Station, Bull. 5* (1914).
4. Michel, C., *Compt. rend. Soc. biol.*, **51**, 422 (1899).
5. Hugounenq, L., *J. physiol. et path. gén.*, **2**, 509 (1900).
6. Camerer, W., and Söldner, G., *Z. Biol.*, **49**, 173 (1902).
7. Givens, M. H., and Macy, I. G., *J. Biol. Chem.*, **50**, p. xxxiv (1922).
8. Schmitz, E., *Arch. Gynäk.*, **121**, 1 (1924).
9. Coons, C. M., and Blunt, K., *J. Biol. Chem.*, **86**, 1 (1930).
10. Macy, I. G., Hunscher, H. A., Nims, B., and McCosh, S. S., *J. Biol. Chem.*, **86**, 17 (1930).
11. Hoffström, K. A., *Skand. Arch. Physiol.*, **23**, 327 (1910).
12. Murlin, J. R., *Am. J. Obst.*, **75**, 911 (1917).
13. Shohl, A. T., *Physiol. Rev.*, **3**, 509 (1923).
14. Roseberry, H. H., Hastings, A. B., and Morse, J. K., *J. Biol. Chem.*, **90**, 395 (1931).
15. Robison, R., *Biochem. J.*, **17**, 286 (1923); Robison, R., and Soames, K. M., *Biochem. J.*, **18**, 740 (1924).
16. Aron, H., and Sebauer, R., *Biochem. Z.*, **8**, 1 (1908).
17. Sherman, H. C., and MacLeod, F. L., *J. Biol. Chem.*, **64**, 429 (1925).
18. Sherman, H. C., and Booher, L. E., *J. Biol. Chem.*, **93**, 93 (1931).
19. Goss, H., and Schmidt, C. L. A., *J. Biol. Chem.*, **86**, 417 (1930).

STUDIES ON THE ANTINEURITIC VITAMIN

I. ON THE USE OF ALBINO MICE AS TEST ANIMALS FOR DETERMINING THE POTENCY OF ANTINEURITIC CONCENTRATES*

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In connection with an investigation dealing with certain aspects of the nutrition of the mouse, we were confronted with the problem of estimating the nutritive value of the daily doses of yeast given as a source of the vitamin B factors. Inasmuch as the mouse requires 200 mg. of yeast daily, which amounts in some cases to one-tenth of the daily food intake, this was a factor which could not be neglected. It was decided, therefore, to prepare concentrated preparations of the antineuritic vitamin.

The next question which arose was the choice of a method for testing our concentrates. Although the procedures in which pigeons and rats are used for this purpose have been well standardized, we thought it worth while to attempt to use mice as test animals. We reasoned that the mouse, on account of its high metabolic rate and its small size, would offer certain advantages for this type of work. In the first place, much less material would be necessary for making the tests. Secondly, we expected that mice would show symptoms of vitamin B deficiency at an earlier date than rats or pigeons. Lastly, it was deemed of interest to repeat, if possible, the work of Jansen and Donath (1) on the isolation of the antineuritic vitamin from rice polishings and to corroborate their findings on another species, the mouse. The observa-

tions of Tsukiye (2), Beard (3), and Bing and Mendel (4) have shown that mice may be used with advantage for vitamin studies.

<div align="center">EXPERIMENTAL</div>

As a source of the antineuritic vitamin we used rice polishings. In the preparation of the concentrates we followed Jansen and Donath's procedure.   Very few changes were made in their method of isolation.   Care was taken that no operation was carried out at a higher temperature than 40°.   All evaporations were made *in vacuo*.   We found that the treatment of the tikitiki extract with 80 per cent alcohol is very useful (Hofmeister (5), Evans and Lepkovsky (6)).   Under these conditions it is possible to eliminate nearly all the contaminating carbohydrate material.   Instead of the acid clay which was employed by Jansen and Donath, we used Lloyd's reagent for the adsorption of the vitamin with excellent results.

The liberation of the vitamin from the adsorbent seems to be a controversial subject.   Jansen and Donath used in their experiments barium hydroxide and reported a recovery of 80 per cent of the vitamin.   Later workers observed considerable losses of the potent factor at this stage of the isolation process.   We refer to the studies of Williams and his coworkers (7), Seidell (8), and Evans and Lepkovsky (6).   Mukherji (9) and Van Veen (10), on the other hand, have confirmed Jansen and Donath's findings.   In our own experiments the recovery was 75 per cent.

In the later stages of the isolation process we followed exactly Jansen and Donath's directions.   The Lloyd's reagent extract was fractionated according to Kossel and Kutscher (11) with silver nitrate and baryta.   Three fractions have been prepared: the first one was precipitated at pH 2 to 4.5, the second at pH 4.5 to 6, and the third at pH 6 to 8.   Inasmuch as the second fraction[1] was shown to contain more than 50 per cent of the vitamin, we used this material for the further purification.   After removing the silver, the potent factor was precipitated with silicotungstic acid, according to Jansen's modification of the first method (12).   The last stages of the concentration were carried out exactly as de-

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[1] From the first and third fractions we isolated a small amount of material in form of the flavianates, but found these to be inactive.   We intend to use flavianic acid in our further work of purification.

scribed by Jansen and Donath, only that instead of platinum chloride we used cadmium chloride, as recommended by Jansen (12).

In the following diagram an outline of the purification process is given.

Rice polishings
|
25 per cent alcohol
|
Concentrated
|
Tikitiki extract
|
80 per cent alcohol
|
Lloyd's reagent
|
Activated Lloyd's reagent (A I) (90 gm. = 25 kilos rice polishings)
|
$Ba(OH)_2$
|
Filtrate (after removal of Ba) (A II) (700 cc. = 25 kilos rice polishings)
|
$AgNO_3$ + $Ba(OH)_2$ at pH 4.5 to 6.5
|
HCl
|
Precipitate
|
Silicotungstic acid
|
$Ba(OH)_2$
|
Absolute alcohol + $CdCl_2$
|
H_2S
|
Impure hydrochloride (A III) (120 mg. = 25 kilos rice polishings)

Physiological Tests

Our experiments were carried out on albino mice bred and reared from an original stock obtained from the zoology department of this University. Young mice were separated from their mothers when they were 3 weeks old. As basal diet for the normal

controls we used the food mixture recommended by Bing and Mendel (4), consisting of purified casein[2] 31 per cent, purified cornstarch 38 per cent, Crisco 24 per cent, and Osborne and Mendel's salt mixture 7 per cent.[3]   In addition, each mouse received separately 200 mg. of dried yeast[4] and 2 drops of cod liver oil daily. In the course of our experiments we found that the cod liver oil could be mixed with the food with apparently the same results.

The basal diet for the test animals was the same as above, except for the yeast, which was substituted by the same amount of autoclaved yeast.   The mice which were receiving the diet lacking the antineuritic factor showed the first symptoms of vitamin B_1 deficiency when they were about 25 days old.   They began to lose weight, became restless, and their fur was unkempt in appearance.   Between the 28th and 35th days, definite symptoms of vitamin B_1 avitaminosis were manifest, such as appreciable loss in weight, the arched back, and darkening of the tail.   At this point we began our tests.

Evaluation of Antineuritic Concentrates

We controlled the process of purification of our vitamin preparation by testing the potency of our concentrates at four stages during the course of the work.

The first tests were made on the tikitiki extract, prepared according to Wells (13), by extracting the rice polishings with 25 per cent alcohol and concentrating this extract to a syrup *in vacuo*. In agreement with Bing and Mendel's observations it was found that 0.03 to 0.04 cc. of the tikitiki extract, given daily, was sufficient for good growth.

For the second test we chose the activated Lloyd's reagent precipitate (Preparation A I).   90 gm. of this precipitate correspond to 25 kilos of rice polishings.   This preparation was fed, mixed with the autoclaved yeast, separately to the mice.   10 mg. of the substance, given daily, were sufficient to cure the animals of polyneuritis and to promote growth.

Inasmuch as several workers have reported unsuccessful at-

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[2] The casein was purified according to the directions of Evans and Lepkovsky (6).

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

[4] The product from the Northwestern Yeast Company was used.

tempts to recover the vitamin from the adsorbent in as good a yield as that obtained by Jansen and Donath, it was deemed necessary to test the potency of the concentrate obtained after removal of the vitamin from the Lloyd's reagent.   700 cc. of this concentrate (Preparation A II) were obtained in the extraction of 25 kilos of rice polishings.   It was found that in all cases prompt and complete cures of polyneuritis resulted from doses of 0.12 cc. of this preparation, and a marked increase in weight occurred.

The last test was carried out with the substance obtained from the cadmium chloride precipitate, after decomposing it with
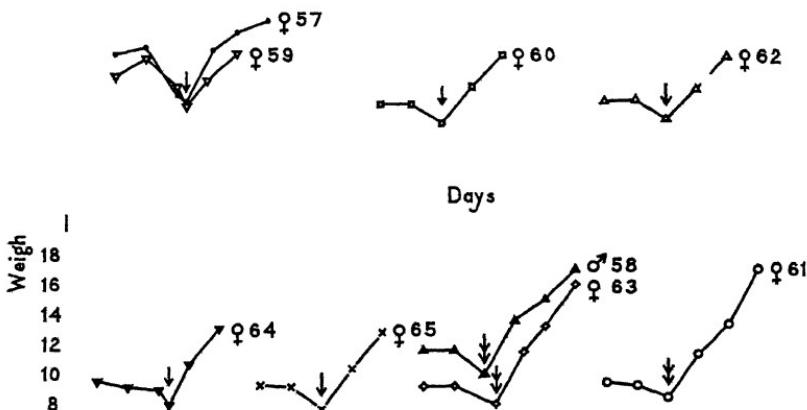
CHART 1.   Growth curves showing the potency of a concentrated preparation of the antineuritic vitamin (Preparation A III).   0.025 mg. of Preparation A III was injected into Mice 57, 59, 60, 62, 64, and 65 at the points indicated by the single arrow; 200 mg. of yeast were fed to Mice 58, 61, and 63 at the points indicated by the double arrow.

hydrogen sulfide.   The filtrate of the cadmium sulfide was evaporated to dryness *in vacuo*.   The residue (Preparation A III) weighed 0.12 gm., corresponding to 25 kilos of rice polishings. This substance was taken up in water, and the solution injected subcutaneously.   The results obtained with this preparation are shown in Chart 1.   It was found that a daily dose of 0.025 mg. of this substance was sufficient to cure the animals of polyneuritis and to promote normal growth.   A few tests were carried out with lower doses of this concentrate, which showed that a daily dose of 0.010 mg. cured the mice of polyneuritis, but in no case, however, did an increase in weight occur.

DISCUSSION

The experiments reported above show that mice may be used with advantage as test animals for the evaluation of antineuritic concentrates.

We have been able to isolate from rice polishings, according to the method described by Jansen and Donath, a potent principle which, in daily doses of 0.025 mg., cures polyneuritis in mice. We believe that we are justified in assuming that the curative and growth-promoting dose of this preparation probably lies between 0.010 and 0.025 mg. Jansen and Donath reported that the substance which they obtained at this stage of the isolation process was curative for rice-birds (*Munia maja*) in daily doses of 0.008 mg. Considering the fact that we are dealing with two different species, we may safely assume that our preparation approaches in its purity the substance obtained by Jansen and Donath.

SUMMARY

1. A potent antineuritic concentrate has been obtained from rice polishings, with Jansen and Donath's method of isolation.

2. This preparation cures polyneuritis and promotes growth in mice in daily doses of 0.025 mg.

3. Our experiments corroborate the findings of Jansen and Donath on still another species, the mouse.

4. Our results show that mice may be used with advantage as test animals for the evaluation of antineuritic concentrates.

BIBLIOGRAPHY

1. Jansen, B. C. P., and Donath, W. F., *Verslag Akad. Wetenschappen Amsterdam*, **29**, 1390 (1926).
2. Tsukiye, S., *Biochem. Z.*, **131**, 124 (1922).
3. Beard, H. H., *Am. J. Physiol.*, **75**, 124 (1926).
4. Bing, F. C., and Mendel, L. B., *J. Nutr.*, **2**, 49 (1929–30).
5. Hofmeister, F., *Biochem. Z.*, **103**, 218 (1920).
6. Evans, H. M., and Lepkovsky, S., *J. Nutr.*, **3**, 353 (1931).
7. Williams, R. R., Waterman, R E., and Gurin, S., *J. Biol. Chem.*, **87**, 559 (1930).
8. Seidell, A., *J. Am. Chem. Soc.*, **44**, 2042 (1922).
9. Mukherji, A., *Indian Med. Gaz.*, **64**, 443 (1929).
10. Van Veen, A. G., *Rec. trav. chim. Pays-Bas*, **50**, 200 (1931).
11. Kossel, A., and Kutscher, P., *Z. physiol. Chem.*, **31**, 165 (1900).
12. Jansen, B. C. P., *Rec. trav. chim. Pays-Bas*, **48**, 984 (1929).
13. Wells, A. H., *Philippine J. Sc.*, **19**, 67 (1921).

FACTORS DETERMINING THE ERGOSTEROL CONTENT OF YEAST

II. CARBOHYDRATE SOURCES*

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It has been known for many years that the sterol content of fungi varies widely. Recently, in studies on this variation under controlled conditions, we found that different species of yeast, grown in a given medium, exhibited decidedly different capacities for the elaboration of ergosterol (1, 2). We shall now consider the influence of cultural conditions, particularly carbohydrate sources, upon a given species.

The carbohydrate experiments are particularly significant, because they give information on the previously obscure mechanism of the formation of ergosterol. Since the sterols are physically associated with the fats, it has been assumed that they are derived from them, or at least that the two have a common genesis. Heiduschka and Lindner (3) held this view on theoretical grounds, and MacLean (4) has proposed to increase the practical yield of ergosterol by growing yeast under conditions favorable to the production of fat. Some actual experiments by Terroine, Bonnet, Kopp, and Vechot (5) failed to demonstrate any quantitative relations between fat and sterol in microorganisms. Heiduschka and Lindner have shown that the yield of ergosterol from a beer yeast varied considerably when the yeast was grown in different media such as malt extract, and wort containing various organic and inorganic additions.

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Our experiments were conducted with a single yeast, grown and harvested under conditions that were always the same except for the source of carbohydrate. The yeast was a wild species isolated from malt by Dr. Robert P. Myers, in 1928. Dr. F. W. Tanner and Mr. E. I. Wheaton have identified it as *Saccharomyces cerevisiæ* of the Frohberg bottom type. It gives a moderately large yield of ergosterol comparatively free from pigmented impurities, and on this account it served as a commercial source of ergosterol until culture methods for the ergosterol-rich *Saccharomyces carlsbergensis* (2) were perfected. For the same reason it has been used for activation by insolation, a thin layer on a traveling belt becoming 100 times more antiricketic than cod liver oil after a few minutes of exposure to sunlight.

Certain difficulties had to be overcome in devising a nutrient solution complete in every respect except carbohydrate. Since purified nutrients never promote vigorous growth of yeast, we employed a wort consisting of crude materials rich in bios, minerals, etc. This was obtained by subjecting dilute beet molasses to a preliminary fermentation in order to remove the sugar. The resulting spent liquor was clarified in a yeast separator, and finally by filtration through diatomaceous earth. Potassium phosphate and urea were added, and thus we had an excellent basal nutrient to which any desired form of pure carbohydrate could be added. The culture medium had the following composition.

Carbohydrate...................................... 50.0 gm.
KH_2PO_4... 1.5 "
Urea.. 3.5 "
Spent liquor, *q.s.*................................... 1000 cc.

It should be noted that the preliminary fermentation was conducted under semianaerobic conditions. Therefore alcohol was produced; and this had to be removed by subjecting the hot solution to a partial vacuum. Presumably also small amounts of glycerol, acids, and other organic substances were developed, and as a result of their presence a slight growth of yeast occurred when the experimental solutions were aerated. However, this would seem far less objectionable than the use of purified nutrients to which an unknown and variable amount of bios would be added with every different sugar.

A sterile culture technique was followed throughout the experiment. Inoculations were made into a liter of nutrient, and the cultures were aerated vigorously. The flasks were kept, in batteries of twelve, in a water bath at 30°. Duplicate inoculations for the same sugar were never run in the same battery. Therefore the close agreement in the duplicate determinations is all the more convincing. The fermentations were continued for exactly 24 hours, and all runs, except in the case of the costly melibiose, were made in duplicate. The yeast was harvested centrifugally, washed with acidified 75 per cent methyl alcohol,[1] and dried *in vacuo* at 75° for 20 minutes.

A portion of the resulting dry yeast was used for a Soxhlet determination of the total lipoid soluble in acetone. The nitrogen content was estimated by the Kjeldahl method.[2] The ergosterol content was determined spectrographically, according to the method previously described (2).

The findings are summarized in Table I, from which it is evident that the carbohydrates fall into three groups with respect to their relation to ergosterol. In the first group are those carbohydrates which did not support any significant growth of yeast. In this group comes the one pentose that we studied, namely xylose. Here also is mannitol, the unfermentable alcohol corresponding to the fermentable mannose. α-Methylglucoside, the simplest ester of glucose, likewise comes here, as do also the dihexose sugars, lactose and melibiose, and the trihexose, melezitose.

With each of the above sugars, and with the control which contained no sugar, the yield of dry yeast amounted to slightly more than 1 gm. This was doubtless due to the inoculum itself, to the by-products of the preliminary fermentation, and to hydrolysis of the sugars during sterilization. Of course, after 24 hours of aeration, the yeast obtained from these media was in a state of starvation, and, as we should expect under such circumstances, the total lipoid was high, amounting in each case to about 5 per cent of the dry weight. It is generally recognized that starving yeast pro-

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[1] Made acid to Congo red by the addition of HCl. The acidified alcohol served to dissolve the precipitate formed by autoclaving the medium. Thus the slight error in yield, noted in our previous work (2), was eliminated.

[2] H. L. Nenneker, analyst.

duces fat at the expense of its protein, and indeed we find that the nitrogen content is low, being approximately from 5 to 7 per cent.

TABLE I

Influence of Carbohydrate Sources on Production of Ergosterol by Yeast

Carbohydrate	Yield of dry yeast per liter of medium	Total acetone-soluble lipoid of dry yeast	N in dry yeast	Ergosterol in dry yeast
	gm.	*per cent*	*per cent*	*per cent*
Control............................	1.1	4.8	6.1	0.5
	1.1			
Xylose............................	1.1	4.6	5.3	0.5
	1.1			
Mannitol..........................	1.1	4.8	5.7	0.5
	1.3			
α-Methylglucoside................	1.3	4.6	7.0	0.5
	1.4			
Lactose...........................	1.2	5.2	6.7	0.4
	1.3			
Melibiose.........................	1.3	4.7	7.1	0.5
Melezitose........................	1.3	4.8	6.9	0.6
	1.4			
Galactose.........................	5.4	1.2	7.4	0.3
	5.9	1.0	7.5	0.3
Mannose..........................	5.6	0.9	8.4	0.3
	5.8	1.1	8.4	0.4
Fructose..........................	4.8	0.9	8.1	0.4
	5.1	1.1	8.1	0.4
Glucose...........................	5.0	1.0	7.8	0.5
	5.1	1.3	8.1	0.6
Galactose-fructose-glucose mixture..........................	4.9	1.0	8.8	0.4
	5.2	1.1	8.7	0.3
Sucrose...........................	5.6	1.1	8.0	0.6
	5.7	1.1	7.9	0.7
Maltose...........................	4.3	1.6	8.0	0.8
	5.2	1.6	7.9	0.7
Maltose (half quantity)............	2.4	2.1	7.7	0.7
	2.5	2.2	7.6	0.8
Raffinose.........................	2.9	2.3	7.7	1.3
	3.0	2.2	7.7	1.1

The ergosterol content of the small amount of yeast obtained was about 0.5 per cent.

The second group of sugars comprises the four fermentable hexoses—galactose, mannose, fructose, and glucose, and also a mixture of three of them—galactose, fructose, and glucose in equal parts. The 24 hour fermentation sufficed to give a good growth of yeast, but it did not put the yeast in a condition of starvation. Accordingly, we note that the yield of dry yeast was approximately from 5 to 6 gm., and the total lipoid content was normal for the species—1 per cent in every case. The nitrogen content was also normal, around 8 per cent.

In this group we find a remarkable variation in the ergosterol yield, galactose giving only 0.3 per cent, mannose and fructose each about 0.4 per cent, and glucose 0.6 per cent. The mixture of galactose, fructose, and glucose gave a quantity of ergosterol similar to the mean of the three sugars separately.

In the third group we list two dihexoses, sucrose and maltose, and a trihexose, raffinose. The maltose fermentations were repeated in one instance, with only a half quantity of carbohydrate. Sucrose and maltose each gave the normal yield of yeast, with a normal fat content and normal nitrogen content. The ergosterol content, however, increased to 0.7 per cent for the sucrose, and 0.8 per cent for the maltose. When only a half quantity of maltose was used, the yield of yeast was reduced to one-half, and as a result of the slight starvation, the lipoid percentage was doubled, and the nitrogen content slightly reduced. Yet the ergosterol content was the same as when the full quantity of sugar was employed.

But the most interesting sugar of all is raffinose. This trisaccharide is hydrolyzed by the yeast enzyme, raffinase, to 1 molecule of fructose and 1 molecule of the non-assimilable disaccharide, melibiose. Consequently the yield of yeast was reduced to 3 gm. and this partially starved. The lipoid content was the same as in the case of the reduced quantity of maltose, namely 2 per cent. In the spent liquor after the fermentation we found the theoretical two-thirds quantity of melibiose polarimetrically. If this melibiose had been hydrolyzed by the raffinase, there would have been available for the yeast equal parts of fructose, glucose, and galactose. Since it was not hydrolyzed, fructose was the only sugar from which the growth of yeast and the elaboration of ergosterol could take place. It is significant that the yeast so produced

contained 1.2 per cent ergosterol, as compared to 0.4 per cent ergosterol when fructose was the carbohydrate source.

It is evident from the data (Table I) that the ergosterol content of yeast bears no relation to the fat content, or, as more exactly stated, it bears no relation to the non-ergosterol lipoid.   Neither is it related to the protein content, nor to the state of starvation or nourishment of the yeast.   It is probable that there are factors as yet unrecognized which affect it, but so far as the present experiment goes, the principal determinant of the ergosterol content of a given species of yeast is the sugar which served as its carbon source.   We must think of ergosterol, therefore, as primarily a product of carbohydrate metabolism.   In one of Terroine's experiments an organism was studied which normally utilized sugar as its source of carbon, but which could in an emergency utilize fat.   Some sterol was produced in either case, but, conceivably, its production from fat involved the intermediation of a carbohydrate.

Returning for a moment to the observation that much more ergosterol is produced from sucrose, maltose, and raffinose than from the monosaccharides into which they are hydrolyzed, we may be permitted a hypothesis in explanation.   The chemical reactions of the hexoses indicate that each of them can exist in several isomeric forms.   It is reasonable to infer, therefore, that the hexoses which are split off from di- and trisaccharides by the enzymes of yeast, exist for a time in forms different from those that we pour out of a bottle.   These modifications, or nascent forms, if one may so call them, appear to be particularly suitable for building up into ergosterol, through the agency of the living yeast cell.

SUMMARY

Yeast was cultured in a basal medium to which various carbohydrates were added.   The ergosterol content was higher with di- and trihexoses than with the monohexoses.   It is assumed that at the instant of cleavage of the more complex sugars, the resulting hexoses exist in forms more readily convertible into sterol than the familiar, stable forms.   These relations, and the fact that the nitrogen content, the non-sterol lipoid content, and the state of nourishment of the yeast bear no relation to its ergosterol content,

indicate that ergosterol is primarily a product of carbohydrate metabolism.

BIBLIOGRAPHY

1. Prickett, P. S., Massengale, O. N., Cox, W. M., Jr., and Bills, C. E., *Proc. Soc. Exp. Biol. and Med.*, **27**, 701 (1930).
2. Bills, C. E., Massengale, O. N., and Prickett, P. S., *J. Biol. Chem.*, **87**, 259 (1930).
3. Heiduschka, A., and Lindner, H., *Z. physiol. Chem.*, **181**, 15 (1929).
4. MacLean, I. S., British patent 295,757 (1928).
5. Terroine, E. F., Bonnet, R., Kopp, G., and Vechot, J., *Bull. Soc. chim. biol.*, **9**, 678 (1927).

STUDIES ON THE TOXICITY OF GOSSYPOL

II. THE EFFECT OF GOSSYPOL UPON THE APPARENT DIGESTIBILITY OF PROTEIN, FAT, AND CARBOHYDRATE AND UPON THE ABSORPTION OF GLUCOSE FROM THE GASTROINTESTINAL TRACT OF THE RAT*

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The toxic action of gossypol when given to a variety of animals by different routes has been briefly discussed in a previous paper (1). One of the most frequently observed conditions in the postmortem examination of rats which died following the oral administration of gossypol was intestinal inflammation. Diarrhea was of common occurrence among the animals which survived. Enteritis of a severe nature was produced by intraperitoneal injections of gossypol and it was observed by Clark (2) that when death was delayed following such injections, intestinal impaction became sufficiently serious to be an indirect cause of death.

Metabolic disturbances in cats following the ingestion of gossypol have been observed by Schwartze and Alsberg (3). These investigators found that gossypol in the diet caused a slight loss of nitrogen both in the feces and the urine. Jones and Waterman (4) found that gossypol inhibited the digestion of protein *in vitro*. These observations bring out the possibility that gossypol in its rôle of an intestinal irritant and inhibitor of proteolytic digestion may interfere with the normal utilization of food. In fact, the inhibitive effect of gossypol has been offered by Jones and Waterman as a tentative explanation of the incomplete digestion of the proteins of cottonseed meal by animals. Macy and Outhouse (5)

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* Published with the permission of the Director of the Oklahoma Agricultural Experiment Station.

observed that during the development of cottonseed meal injury in dogs the nitrogen balance became negative.   In the above studies a slight loss in body weight occurred simultaneously with the decreased nitrogen absorption.

In previous digestion experiments carried out with rats during short feeding periods (6) it was found that the amount of gossypol which the animals would tolerate without loss of appetite and subsequent decrease in weight did not materially decrease the apparent digestibility of the protein in the diets.   No experiments have been reported which show the effect of gossypol upon digestion of food constituents other than protein or upon intestinal processes in general.   A few experiments (6, 7) have been made to compare the digestibility of autoclaved cottonseed products which contain very little if any gossypol with that of the raw and commercial products which contain varied amounts.   The possible influence of gossypol upon digestibility was obscured in these studies by the marked nutritive changes produced in the products as a result of the autoclaving process.

It is the purpose of this paper to report the results of digestion and absorption experiments carried out on rats receiving moderate amounts of gossypol.

EXPERIMENTAL

In these experiments acute gossypol poisoning which might result in intestinal lesions (5) or chronic poisoning sufficiently serious to be detectable by outward manifestations of injury was not desired.   To this end gossypol was incorporated in experimental diets in amounts which would not seriously affect the appetite of the animals or cause them to lose weight.   The gossypol additions were made by means of an ether extract of cotton seeds of known gossypol content.   Such a procedure has been employed for the preparation of gossypol diets in previous studies (1, 8) and found satisfactory.   To insure against the decomposition of the gossypol or other changes which may occur on long standing, the extracts were made up fresh each week and the ether removed *in vacuo*.   A portion of the extracts was reserved for gossypol determinations.

Digestion experiments were carried out in the usual manner at frequent intervals during an experimental period of 90 days.   The

rate of absorption of glucose by the gossypol-fed animals and normal controls was then determined by methods described in a later part of this paper.

I. Digestion Studies

The experimental animals were adult white rats weighing between 120 and 250 gm. They were evenly distributed into two groups according to sex and weight. Group I, which served as a control, received 3 cc. of refined cottonseed oil per 100 gm. of food. Group II received an equivalent amount of crude cottonseed oil containing 60 mg. of gossypol. Later Group III, which received twice this amount of gossypol, was introduced. All the

TABLE I

Composition of Diets Used in Digestion Studies

Diet for group No.	I	II	III
Ground wheat, *gm.*	64.7	64.7	64.7
Skimmed milk powder, *gm.*	31.0	31.0	31.0
Sodium chloride, *gm.*	1.3	1.3	1.3
Cod liver oil, *gm.*	3.0	3.0	3.0
Refined cottonseed oil, *cc.*	3.0		
Crude cottonseed oil (approximate), *cc.*		3.0	6.0
Gossypol, *mg.*		60.0	120.0

animals received the same basic diet, the components of which together with the gossypol and oil additions are shown in Table I.

In order that only fresh extracts of cottonseed should be used, it was necessary to prepare new diets about every 7 days. Analysis showed only slight differences in their composition, the greatest variation being caused by the omission of cod liver oil in the first diets used. From results of previous studies (9) conducted by one of the authors, it is not believed that this omission affected digestibility by the difference in the amount of fat-soluble vitamins supplied. The results of the analyses of the diets used during the various periods are shown in Table II.

The animals in groups of two or three were kept in false bottom screen cages. During the digestion experiments they were placed in specially constructed metabolism cages (6) which permitted a separation of feces and urine and an accurate measurement of the

food intake.   The animals on gossypol diets frequently developed a diarrhea which usually continued for several days.   Whenever this condition developed during a digestion trial, the excreta collections were discarded and the animals were replaced by a new group.

The diets and feces were analyzed for protein, fat, ash, moisture, and fiber and the carbohydrates (nitrogen-free extract) calculated

TABLE II

Comparison of Diets Used during Various Digestion Periods

Group No.	Digestion period	Average per cent in diet of			
		Protein	Fat	Fiber	Carbohydrate
I	1–5	19.7	3.3	1.8	61.6
	7–9	19.3	6.2	1.8	59.9
II	1–4	19.6	3.2	1.9	61.4
	6–9	19.7	6.6	1.7	59.2
III	1–4	19.0	5.8	1.9	60.4
	6–9	16.4	9.3	1.9	59.3

by difference.   The coefficients of digestibility of protein, fat, and carbohydrate were calculated as follows:

Coefficient of digestibility =

$$\frac{\text{Amount ingested} - \text{amount in feces}}{\text{Amount ingested}} \times 100$$

A few experiments were carried out to determine the rate of passage of food through the digestive tract of gossypol-fed and normal animals.   Carmine markers were used for this and the time which was required for the dye to appear in the feces noted. After a few preliminary trials it was found advantageous to administer a suspension of carmine in water by means of a small stomach tube and syringe.   This was done late in the evening when the animals were active and eating readily.   Feces collections were made by placing the individual cages over large funnels lined with filter paper to absorb the urine and to allow the feces to collect in a test-tube attached to the stem of the funnel.   The

time required for the passage of food as indicated by the appearance of the dye in the feces was approximately 11 hours. No differences were noted between the gossypol-fed and the control animals.

The data obtained in the digestion experiments are shown in Table III. The laboratory numbers presented in the second column of Table III to identify the rats indicate the number of animals employed in each period, and disclose the number of times the same animals were on experiment.

At no time during the digestion studies did the animals in any of the groups lose weight, and although the gains in weight were irregular this was to be expected since all the animals had passed the stage of rapid growth. The animals which received the extracts of gossypol made practically the same total gain as did the control animals which received an equivalent amount of cottonseed oil but no gossypol. The average daily gain varied from 0.65 to 1.25 gm. The average daily food intake of the animals in Groups I, II, and III was 13.12, 11.65, and 9.06 gm., respectively. Therefore, the approximate amount of gossypol consumed daily by the animals in Groups II and III was 7 and 11 mg., respectively. Young rats on diets containing this much gossypol are unable to grow and usually die in less than 75 days.

In evaluating the results presented in Table III it is of importance to note that the average coefficients of digestibility of protein, fat, and carbohydrate obtained with the animals in Groups II and III, whose diets contained 0.06 and 0.12 per cent gossypol respectively, were approximately the same as those obtained with the control animals in Group I. No significant changes occurred in digestibility as measured by these coefficients, even after the animals had been on these diets for over 70 days. This result seems conclusive in spite of the fact that the action of gossypol tends to produce diarrhea and intestinal inflammation. The apparent increase in the digestibility of fat which occurred after Period 5 is not considered significant inasmuch as it was probably caused by the addition of fat to the diet.

These facts suggest that there is no serious impairment of the digestive functions of the intestine as a result of the daily ingestion of such moderate amounts of gossypol as were used in this study. The results do not preclude the possibility that gossypol decreases

TABLE III

Summary of Results Obtained in Digestion Studies

	Period No.	Rat No.	Days on diet	Length of digestion period	Food consumed	Weight of feces	Coefficients of digestibility		
							Protein	Fat	Carbohydrate
				days	*gm.*	*gm.*			
Group I	1	3a	5	7	105.58	13.72	80.0	83.7	91.2
	2	3a, 3c	13	7	187.96	22.92	82.9	83.9	92.1
	2	2a, 2b	13	7	197.77	27.38	81.4	82.0	89.8
	4	7a, 7c	23	5	128.44	14.61	82.4	83.2	92.6
	5	2a, 2b	37	6	142.54	16.60	83.1	79.6	92.5
	7	2a, 2b	52	6	188.28	21.44	83.9	85.0	92.6
	9	2a, 2b	79	6	101.92	14.05	80.0	90.1	91.0
Average...							81.5	83.9	91.7
Group II	1	4b	5	7	84.71	10.26	82.3	79.4	91.6
	1	5a	5	7	108.82	12.92	82.0	81.3	92.3
	2	5b, 5c	13	7	167.79	19.09	84.9	81.3	92.1
	4	6b, 6c	23	7	100.35	13.28	81.4	76.5	91.1
	4	4a, 4c	23	7	195.03	24.28	81.5	78.6	91.8
	6	4a, 4c	44	7	190.66	16.60	82.4	89.1	92.8
	6	4b, 5a	44	7	152.26	17.46	82.0	88.6	92.7
	6	5b, 5c	44	7	174.98	19.93	83.7	89.1	92.6
	7	6b, 6c	52	7	135.33	15.06	83.2	90.1	92.7
	8	5a, 5b	60	6	148.91	18.13	80.7	90.7	92.2
	8	6a, 6b	60	6	129.75	14.74	81.7	92.6	92.7
	9	4b, 4c	72	7	159.28	18.91	82.6	88.0	92.2
Average...							82.4	87.6	92.2
Group III	1	9a, 9c, 9d	6	5	120.80	14.13	81.6	87.0	92.3
	1	8a, 8b	6	3	54.36	5.51	85.7	90.6	92.8
	1	8c, 8d	6	6	95.74	10.42	85.8	85.3	93.3
	2	9a, 9c, 9d	8	5	84.36	10.08	83.5	94.0	90.4
	4	8c	21	6	80.31	8.97	85.1	88.4	92.1
	4	8b, 8d	29	6	101.46	13.14	80.1	90.8	91.9
	5	8c, 8d	35	4	101.46	10.94	84.7	92.2	92.6
	6	8b	41	7	60.64	6.43	85.7	91.2	92.8
	6	8c	41	6	89.56	8.43	87.5	89.6	93.7
	7	8d, 8c	51	5	104.76	9.75	87.3	95.7	92.8
	9	8a	71	10	90.11	12.78	74.9	92.9	89.2
	9	8b	71	10	85.01	7.35	86.7	95.0	93.4
	9	8c	71	10	83.18	10.40	79.7	93.5	90.4
Average...							83.5	91.2	92.0

the rate of digestion of the above food constituents or that in excessive amounts it may prevent their complete breakdown into absorbable products. However, large amounts of gossypol influence growth markedly and lead to a general breakdown in the health of the animals. With the advent of this condition, the results of digestion experiments, which at their best are only gross measurements of food utilization, would be subject to much criticism.

There was a noticeable decrease in the food intake of the animals which received 0.12 per cent gossypol, and in the time which elapsed between the completion of the digestion studies and the beginning of the absorption studies (less than 20 days) some of these animals succumbed. Previous to this, one died of an ear infection. In the light of past experience with rats on gossypol diets, it seems reasonable to assume that 0.12 per cent is close to the maximum amount of gossypol which rats will consume without relatively early injury.

II. Absorption Studies

Various investigators have reported the effects of fatal doses of gossypol upon the gastrointestinal tract. Macy and Outhouse (5) reported the presence of lesions in the intestines of dogs following acute gossypol poisoning produced by the oral administration of gossypol acetate. Macroscopic examination of the intestines revealed the presence of deep hemorrhagic areas and a sloughing off of the mucosa. A large amount of yellow fluid was present in the abdominal cavity of the animals and there was extreme congestion of all splanchnic organs. Macy and Mendel (10) and Macy and Alter (11), after feeding cottonseed meal and cottonseed kernels to various animals, observed injury to the intestinal wall which ranged from congestion and small hemorrhages to extensive necrosis of the mucosa and deeper layers. The possibility of gossypol affecting the musculature of the intact intestine is indicated by the results of Schwartze and Alsberg (3) who found that a dilution of gossypol of 1:5000 produced definite paralysis of the isolated intestine of the rabbit. At greater dilution the results became less definite.

Although physiological amounts of gossypol produced no change in intestinal activity capable of detection by the digestibility

studies described in the first part of this paper, there was the possibility of injury which would be revealed by changes in the rate of absorption from the intestine.   Glucose was chosen as a suitable substance for the study of absorption since methods were available for its determination in the presence of other soluble material in the intestinal tract and because its absorption from the intestine at a fairly uniform rate has been established.   Cori's method (12) was followed for this work.   The method briefly stated consists in administering by stomach tube a known amount of glucose to rats which have fasted for 24 or 48 hours.   After a definite period of time, usually less than 4 hours, the rats are killed, and the gastrointestinal tract is removed, opened, and washed free of glucose. The amount of glucose absorbed is calculated from the amount administered and the amount remaining in the tract.

In these studies the animals were fasted for 48 hours.   2 cc. of a 50 per cent glucose solution were administered to the animal under light ether anesthesia.   The stomach tube was rinsed with 0.5 cc. of water.   At the same time and in the same manner, 2 cc. of the sugar solution were discharged into each of two 500 cc. volumetric flasks.   These solutions, which served as a control to determine the amount of sugar administered to the animals, were made up to volume and their sugar content determined.   At the end of 3 hours the animals were chloroformed.   The gastrointestinal tract after ligation at the esophagus and anus was removed, opened, and washed free of sugar with about 400 cc. of hot water.   The resulting solution was clarified with colloidal iron upon the addition of a few drops of saturated sodium sulfate solution and made up to 500 cc.   The glucose was determined by Bertrand's method (13). A few animals were chloroformed immediately following the sugar administration and the per cent of sugar recovered was used as a further check upon the accuracy of the procedure.

The absorption coefficient (gm. of sugar absorbed per 100 gm. of body weight per hour) and the per cent of sugar absorbed by gossypol-fed and normal animals are shown in Table IV.   The coefficients so obtained are somewhat lower than those reported by Cori and approximate those which Pierce, Osgood, and Polansky (14) report for normal "Penn State" rats.

An inspection of Table IV shows that the absorption coefficients obtained with the rats which had subsisted for approximately 100

TABLE IV

Summary of Data Obtained in Absorption Studies

Group I received cottonseed oil, Group II 0.06 per cent gossypol, and Group III 0.12 per cent gossypol.

	Days on diet	Body weight after fasting 48 hrs.	Loss of body weight in 48 hrs.	Glucose fed	Total glucose absorbed	Absorption coefficient	Glucose absorbed
		gm.	per cent	gm.	gm.	gm.	per cent
Group I. Absorption period 2 hrs.	101	168	8.7	0.998	0.413	0.122	41
	101	182	7.1	0.998	0.476	0.130	48
	101	193	6.8	0.998	0.278	0.072	28
Average...						0.108	39
Group I. Absorption period 3 hrs.	103	176	8.3	0.998	0.678	0.128	68
	107	232	8.0	0.998	0.464	0.066	46
	103	237	7.1	0.998	0.638	0.090	64
	103	242	6.3	0.998	0.740	0.102	74
	107	270	7.2	0.998	0.555	0.069	56
Average...						0.091	62
Normal. Absorption period 3 hrs.		117	7.7	0.998	0.442	0.126	44
		131	7.7	0.989	0.389	0.099	39
		169	8.2	0.989	0.723	0.143	73
		144	10.0	0.989	0.490	0.113	50
		147	7.5	0.989	0.723	0.164	73
		145	12.7	0.989	0.670	0.154	67
		176	7.4	1.044	0.767	0.145	73
		180	10.0	1.044	0.696	0.129	66
		189	7.8	1.044	0.530	0.094	51
		203	7.7	1.044	0.768	0.126	73
Average...						0.129	63
Group II. Absorption period 3 hrs.	110	152	7.9	1.044	0.634	0.139	61
	110	153	9.5	1.044	0.640	0.139	61
	103	179	10.1	0.998	0.561	0.104	56
	110	188	8.7	1.044	0.634	0.112	61
	108	199	7.0	0.998	0.793	0.132	79
	103	240	6.6	0.998	0.571	0.079	57
	103	250	5.7	0.998	0.609	0.081	61
	103	260	6.5	0.998	0.538	0.070	54
	108	275	5.2	0.998	0.740	0.090	74
Average...						0.105	63

TABLE IV—*Concluded*

	Days on diet	Body weight after fasting 48 hrs.	Loss of body weight in 48 hrs.	Glucose fed	Total glucose absorbed	Absorption coefficient	Glucose absorbed
		gm.	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*
Group III. Absorption	77	160	12.6	0.998	0.378	0.079	37
period 3 hrs.	34	165	12.7	0.998	0.538	0.109	53
	34	169	11.1	0.998	0.464	0.091	46
	77	174	7.4	0.998	0.139	0.027*	14*
	33	182	10.3	0.998	0.676	0.124	64
Average..						0.101	50

* Value not included in average.

days on a diet containing 0.06 per cent gossypol were approximately the same as those obtained with rats receiving the same basic diet without the gossypol supplement.   Coefficients of about the same order were obtained with the group of rats which received 0.12 per cent gossypol, although it should be noted that some of the animals in this group were on the experimental diet for only about 35 days.   The average absorption coefficient calculated for the above three groups is 0.101.   For rats that had been reared on a stock diet the values were somewhat higher, the average coefficient for this group being 0.129.   The significance of this higher value is not clear.   Possibly the higher coefficient obtained with the normal group is to be related to the weight of the animals, a factor which is discussed later.   A comparison of the coefficients obtained with the various groups of animals would seem to indicate that gossypol ingestion produced slight if any intestinal disturbance which resulted in gross injury to the structures of the intestine concerned with absorption.

When the percentage of sugar absorbed rather than the coefficient of absorption is used as the basis for comparison, the slower absorption of glucose by the animals in Group III is made apparent. With the exception of a few cases the percentage of sugar absorbed by the normal animals does not show much variation although there are wide differences in the weights of the animals.   This holds true in many instances for the animals in Groups I and II. Sufficient data have not been obtained to test the validity of expressing the rate of sugar absorption on this basis and for that reason great emphasis should not be placed on these figures.

The fact that the calculated coefficients of absorption are lower than those reported by Cori (12) for normal rats justifies brief comment. Cori's average coefficient of 0.178 was obtained with young rats. This investigator states that rats between 2 and 3 months of age and weighing from 120 to 180 gm. are most suitable for a study of glucose absorption. He further points out that a proportionality between the amount of glucose absorbed and the body weight was found in rats which were in a period of active growth, a fact which may not be valid for fully grown rats where variations in the fat deposits and other factors may play a rôle. As previously stated, mature rats approximately 6 months old were used in this experiment. Most of them had ceased active growth and had deposited considerable fat. It is believed that these facts will explain in part the lower coefficients presented here.

The use of ether anesthesia during the administration of the glucose may have contributed an element of error in these experiments, although this method was used with apparent success by McCance and Madders (15) whose results for xylose and arabinose are even higher than those reported by Cori.

Errors in technique may have been introduced in the administration of the sugar. Low results might be taken to indicate that a larger amount of sugar was administered than was calculated, although it seems unlikely that more sugar would be delivered into the stomach of the rat than into the flasks which served as controls. The facts that the two standards did not show an appreciable difference in their sugar content and that the sugar solutions were of approximately the same strength from day to day, as shown in the fourth column of Table IV, attest the accuracy with which the sugar additions were made. Furthermore, it was found that over 99 per cent of the calculated amount of sugar administered could be recovered from the animals which were killed immediately following the administration of the sugar. These results are therefore considered relative if not absolute and serve for the purpose of comparison although they may not be reproducible with rats of different ages under other experimental conditions.

<div align="center">SUMMARY</div>

The effect of the ingestion of physiological amounts of gossypol upon digestion and absorption in rats has been studied. Digestion

experiments were performed at intervals over a period of 90 days. At the end of this period, the rate of absorption of glucose was determined.

The apparent coefficients of digestibility of protein, fat, and carbohydrate determined with the animals which received 0.06 and 0.12 per cent gossypol in their diets, respectively, remained constant over the entire period and were practically the same as the coefficients obtained with the control animals which received cottonseed oil. It would appear, therefore, that the ingestion of gossypol by rats in amounts of 7 and 11 mg. daily does not lead to an impairment in digestion, as would be evidenced by small coefficients of digestibility.

Slight evidence of changes in the rate of intestinal absorption is brought out by the absorption studies. It was found that within a given period of time a smaller per cent of glucose was absorbed by the rats which had received diets containing 0.12 per cent gossypol than by the controls. A comparison of the coefficients of absorption, however, fails to confirm this evidence.

It is concluded that the malady commonly referred to as chronic gossypol poisoning may not be directly attributed to nutritional failure occasioned by impaired digestion and absorption.

BIBLIOGRAPHY

1. Gallup, W. D., J. Biol. Chem., **93**, 381 (1931).
2. Clark, E. P., J. Biol. Chem., **75**, 725 (1927).
3. Schwartze, E. W., and Alsberg, C. L., J. Agric. Research, **28**, 191 (1924).
4. Jones, D. B., and Waterman, H. C., J. Biol. Chem., **56**, 501 (1923).
5. Macy, I. G., and Outhouse, J. P., Am. J. Physiol., **69**, 78 (1924).
6. Gallup, W. D., J. Biol. Chem., **76**, 43 (1928).
7. Gallup, W. D., J. Agric. Research, **42**, 665 (1931).
8. Schwartze, E. W., and Alsberg, C. L., J. Agric. Research, **28**, 173 (1924).
9. St. Julian, R. R., and Heller, V. G., J. Biol. Chem., **90**, 99 (1931).
10. Macy, I. G., and Mendel, L. B., J. Pharmacol. and Exp. Therap., **16**, 345 (1921).
11. Macy, I. G., and Alter, N. M., Am. J. Physiol., **55**, 304 (1921).
12. Cori, C. F., J. Biol. Chem., **66**, 691 (1925).
13. Mathews, A. P., Physiological chemistry, New York, 4th edition, 952 (1925).
14. Pierce, H. B., Osgood, H. S., and Polansky, J. B., J. Nutr., **1**, 247 (1928–29).
15. McCance, R. A., and Madders, K., Biochem. J., **24**, 795 (1930).

THE INACTIVATION OF CRYSTALLINE INSULIN BY CYSTEINE AND GLUTATHIONE*

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In some earlier work on insulin (1, 2) we have shown that the sulfur of insulin existed mainly if not entirely in the disulfide form, and further that the major portion of the disulfide content could be accounted for by cystine. In continuation of this study of the sulfur of insulin we felt it would be worth while to study the behavior of the reduced or sulfhydryl form of insulin. If the disulfide grouping alone could be reduced, it would give much information as to whether the disulfide linkage as such were necessary to the action of insulin.

It has been shown by many different workers that the reduction of various insulin preparations destroyed their activity. Practically no such work has been carried out on crystalline insulin. The reducing agents used in the above work, however, were quite vigorous ones and there could be no justification for believing that no other part of the molecule besides the disulfide grouping had been reduced.

There is some reason to believe that the action of cysteine might prove to be fairly specific for reducing the disulfide grouping. Mirsky and Anson (3) applied somewhat the reverse reaction to proteins in devising a method to measure the sulfhydryl groups in denatured proteins. They oxidized the sulfhydryl groups by using an excess of some disulfide. They fully realized that the

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validity of their method depended on whether only the sulfhydryl groups of the protein reacted with the disulfide and presented evidence in support of this. By the mass action of the large amount of the added disulfide, the sulfhydryl form was converted to the disulfide. They also found that the addition of large amounts of sulfhydryl compound to the disulfide form of the protein shifted the equilibrium back again and the disulfide groups of the protein were converted to the sulfhydryl again.

The action of cysteine on insulin was therefore studied. Our attention was also drawn to glutathione, especially from the standpoint of a possible relationship between this compound and insulin in the animal body.

EXPERIMENTAL

The insulin used throughout these experiments was crystalline. It was prepared by Abel's brucine method according to the directions given by du Vigneaud, Jensen, and Wintersteiner (1).

The blood sugar determinations in all these experiments were carried out by the method of Hagedorn and Jensen (4). All the determinations were performed in duplicate. The blood samples were taken from the marginal ear vein of the rabbit 1.5 and 3 hours after the injection of the insulin in some of the experiments, while in others they were taken at the 2 hour point.

The cysteine hydrochloride was prepared both by the method of du Vigneaud, Audrieth, and Loring (5), involving the reduction of cystine by metallic sodium in liquid ammonia, and by the usual tin reduction method. No difference in the behavior of the cysteine prepared by these two methods towards insulin was noticed.

The glutathione was obtained from the Eastman Kodak Company and analyzed by the method of Mason (6). Close to theoretical values for the reduced form were obtained.

The cysteine hydrochloride solutions were made up to contain 20 mg. of cysteine per cc. The solutions were kept in an automatic burette in an atmosphere of nitrogen, under which conditions the cysteine could be kept in the reduced form. Analyses from time to time showed that practically no reoxidation took place over long periods of time. The nitrogen used for this and for other work in this investigation was purified by passing it through a

large combustion tube containing reduced copper wire heated to a red heat in a combustion furnace.

Cysteine

In the experiments devised to study the effect of cysteine on insulin a neutralized solution of insulin was added to a neutralized solution of cysteine hydrochloride. The resulting solutions were always checked again for neutrality towards litmus. The concentration of the solutions was so arranged that the final solution contained 0.5 mg. of insulin per cc., the cysteine concentration varying in the various experiments. The solutions were then allowed to stand the desired length of time in an atmosphere of nitrogen. Injections were then made of the insulin-cysteine solution. Suitable control experiments demonstrated that the cysteine in the concentrations injected did not appreciably affect the blood sugar. This was done in preference to isolating the reduced insulin before injecting in order to test the activity at a given time and particularly to remove the possibility of reoxidation which might occur in the handling of the product during isolation. The reduced insulin is extremely readily reoxidized.

The results shown in Table I demonstrate convincingly that insulin is inactivated by cysteine in neutral solution. The ratio of insulin to cysteine in these experiments varied from 1:1 to 1:40. The insulin was inactivated within 0.5 hour with the stronger concentrations, but with a 1:10 ratio a longer period of contact was required. When a ratio of 1:4 is used, 12 hours are necessary to insure inactivation; in fact, even with this length of time there is an indication of a slight residual activity. A comparison of these values with those of a 1:2 ratio shows that we were just on the border-line. It is rather an interesting point that these lower ratios inactivate insulin if given sufficient time. It is worth pointing out that in these as well as in the other experiments 0.5 mg. of insulin was injected per kilo of body weight of the rabbit which is 10 or more times the ordinary convulsive dose for crystalline insulin.

We have made many attempts to restore the potency of the insulin inactivated by cysteine but in all instances so far these

attempts have not been successful. This phase of the work as well as the loss in the ability of the inactivated material to yield a heat precipitate is considered in greater detail in that part of this report which deals with the inactivation of insulin by glutathione.

Since iron acts as catalyst in the oxidation of cysteine to cystine, it was thought possible that the reaction between insulin and cysteine might also be influenced by iron. The use of cysteine

TABLE I

Inactivation of Crystalline Insulin by Cysteine

0.5 mg. of insulin was injected per kilo of body weight.

Rabbit No.	Weight	Ratio insulin: cysteine	Time	Blood sugar, mg. per 100 cc.		
				Normal	1½ hrs .	3 hrs.
	kg.		hrs.			
22	2.8	1:40	30	108	109	120
7	2.4	1:40	0.5	108	117	117
59	2.3	1:20	1	92	78	90
15	2.6	1:20	0.5	109	96	105
54	2.7	1:10	14	126	113	128
60	2.2	1:10	1	96	63	75
20	2.3	1:10	0.5	130	57	*
22	2.5	1: 4	12	117	113	112
28	1.9	1: 4	1	119	115	105
30	2.7	1: 4	0.5	103	45	*
20	1.9	1: 2	12	131	94	96
22	2.8	1: 2	12	113	74	90
31	2.3	1: 2	0.5	122	88	87
16	3.0	1: 2	0.25	116	*	
15	2.6	1: 1	12	115	53	*
31	2.3	1: 1	1	110	*	
10	2.1	1: 1	0.5	113	56	*

* Convulsions.

hydrochloride washed with acetone to free it from iron salts leads to results no different from those reported above. Furthermore a few experiments were carried out in which sodium cyanide was added in amounts that have already been shown to be sufficient to inhibit the catalytic action of iron on the cysteine-cystine reaction. The results demonstrated the independence of the reaction from such catalysis.

Glutathione

As an example of another sulfhydryl compound, and particularly because of its presence in the blood and body tissues, the action of glutathione on insulin was therefore studied. It was found that insulin was inactivated in neutral solution by this sulfhydryl tripeptide. A larger amount of glutathione than cysteine was required. In proportion to the sulfhydryl content

TABLE II

Inactivation of Insulin by Glutathione

Rabbit No.	Weight	Dose per kilo	Ratio insulin: glutathione	Time	Blood sugar, mg. per 100 cc.	
					Normal	1½ hrs.
	kg.	*mg.*		*hrs.*		
108	2.12	0.5	1:40	1.5	117	107
111	2.32	0.5	1:40	1.5	101	100
112	2.27	0.5	1:20	1.5	124	119
61	2.34	0.5	1:20	1.5	138	133
55	2.4	0.5	1:10	20	117	125
54	2.2	0.5	1:10	20	121	123
62	2.6	0.25	1:10	20	105	107
113	2.33	0.5	1:10	1.5	132	130
41	2.15	0.5	1:10	1.5	127	127
41	2.3	0.5	1:10	0.75	93	83
59	2.3	0.1	1:10	0.75	79	79
53	2.7	0.5	1: 4	30	124	61
56	2.0	0.5	1: 4	16	119	72
46	2.3	0.25	1: 4	16	114	65
40	2.1	0.5	1: 2	30	86	54
49	2.4	0.5	1: 2	16	123	87
48	2.5	0.25	1: 2	16	110	69

approximately 2.5 times as much glutathione as cysteine should be required. From Table II it will be seen that a 1:10 ratio of insulin to glutathione results in the inactivation of the insulin within 1.5 hours. These experiments were carried out similarly to those on cysteine, the final concentration of insulin here being 1 mg. per cc. The action of glutathione on insulin is comparable to that of cysteine and no specificity was apparent. The action seems to be due simply to the mass action of the sulfhydryl on the disulfide groups.

Numerous attempts were made to reactivate the insulin that had been inactivated by cysteine or glutathione. In no case have we been able to regain the activity. It seems to be irreversibly destroyed. One series of experiments might be advantageously reported which not only brings out the negative results from attempts to reactivate the material by reoxidation, but also demonstrates that the ability to form a heat precipitate is also lost through inactivation by cysteine and glutathione, and brings out other properties of the inactivated material.

In one of these experiments 43.5 mg. of crystalline insulin were dissolved in 2 cc. of 0.1 N HCl and 3 cc. of water. The mixture was then neutralized with 0.1 N NaOH and diluted to 8.7 cc. 8 cc. of this solution were added to 25 cc. of a neutralized glutathione solution containing 400 mg. of glutathione. Enough water was then added to make a volume of 40 cc. and the solution kept in an atmosphere of nitrogen for 1.5 hours.

Some of the solution was used at this point for injection into rabbits to test the activity; it showed no activity when injected in 0.5 mg. per kilo doses. The rest of this sample was then used for the reoxidation experiment. Oxygen was allowed to pass through the solution until the sulfhydryl test was completely negative. The insulin was oxidized either directly or through the medium of the reoxidized glutathione. During the process a finely divided precipitate formed. For the rabbit tests on this reoxidized material the solution was stirred up and portions of the entire mixture injected. The results show that no reactivation had resulted. The precipitate mentioned was acid-insoluble and accounted for practically all the insulin originally present in this portion of the sample.

Another sample of insulin inactivated by glutathione was utilized to test whether the inactivation destroyed the property of insulin of yielding an insoluble hydrochloride in 4 per cent hydrochloric acid, the so called insulin hydrochloride. To the insulin-glutathione solution 20 per cent HCl was added until the concentration was 4 per cent. A precipitate was obtained in amount equal to the control which contained no glutathione. The tube was then centrifuged and the precipitate washed with 4 per cent HCl until the wash liquid no longer yielded a positive nitroprusside test. The precipitate was dissolved in ammonium

hydroxide and tested with nitroprusside. A pink color was obtained, while the control with the same amount of ammonium hydroxide gave a negative test.

In some other work on insulin we have found that crystalline insulin is soluble in dry liquid acetamide and in liquid ammonia. Samples of the inactivated reoxidized insulin were also tested for their solubility in acetamide. A 5 mg. sample inactivated as above by a 2 hour contact with a 1:10 ratio of glutathione was precipitated with dilute acid to the point of maximum precipitation, centrifuged, washed with water, alcohol, and ether. To the dry precipitate which was now in the reoxidized but still inactive form melted acetamide was added. The precipitate was only very slightly soluble, whereas the control sample of insulin dissolved entirely.

A reaction of insulin which seems to offer the possibility of being characteristic of insulin is the formation of the heat precipitate. It was reported by du Vigneaud, Geiling, and Eddy (7) that crystalline insulin dissolved in 0.1 N HCl yields a flocculent precipitate when heated in a boiling water bath. It was also noted that the precipitate is insoluble in dilute acid and that it recovers its solubility in this medium after solution in alkali and immediate acidification. We felt it would be very interesting to see whether the inactivated material would yield a similar heat precipitate. To an 8 cc. glutathione-insulin solution prepared as described above containing 8 mg. of insulin, dilute HCl was added until the point of maximum precipitation was reached and the mixture centrifuged. The precipitate was then washed with ice water. In contrast to the acid-insoluble material which forms after longer treatment with glutathione this material is acid-soluble and can be precipitated again at the isoelectric point. After it had been freed from glutathione by washing the material, it was dissolved in 1 cc. of 0.1 N HCl and placed in a boiling water bath. No heat precipitate formed. A control sample put through the same manipulation except for the addition of glutathione yielded the typical heat precipitate. This loss in ability to form the heat precipitate has also been repeatedly verified with the material inactivated by cysteine.

Other experiments brought out the fact that the acid-insoluble

material reported above was partially convertible to an acid-soluble form by dissolving it in very dilute alkali and immediately acidifying. This reversal of solubility brings to mind the reversal of solubility of the heat precipitate on treatment with alkali. But in spite of the conversion to an acid-soluble form the material did not yield a heat precipitate and was inactive.

<div align="center">DISCUSSION</div>

The results obtained above show without question that insulin is inactivated by even such reducing agents as cysteine and glutathione. The work also indicates that only the disulfide grouping has been reduced under these conditions, although it cannot be considered that this point is definitely proved. It is possible that some other grouping besides the disulfide is being reduced, causing the inactivation, and that the reduction of the disulfide is simply an accompanying reaction without influence on the potency. However, from the lack of any evidence in this direction it seems unnecessary to make such an assumption. We feel instead that the evidence actually points towards the reduction of the disulfide grouping as the cause of the inactivation and that the sulfhydryl form of insulin is inactive.

The consistently negative results of our attempts to reactivate by reoxidation the insulin inactivated by such mild reducing agents are striking. If only the disulfide grouping had been reduced, then we are forced to conclude that reoxidation does not yield again the same disulfide compound. In consideration of the large amount of cystine in insulin and the high molecular weight of insulin, there must be many cystine molecules within the insulin molecule. If that be true, then on reduction we would have many fragments each with one or more sulfhydryl groups which on reoxidation we would have no justification to assume would reunite in exactly the same way as in the original insulin.

The disulfide linkage, *per se*, is however surely not responsible for the insulin action. That is clearly shown by the above experimental work as well as by the work of Blatherwick (8) and Freudenberg (9) and their coworkers. This work does not preclude there being present however a particular arrangement of the disulfide within the molecule responsible for the activity. What we wish to submit is that more than merely a disulfide grouping is

necessary to the action but that the disulfide is nevertheless necessary to this action.   Upon reoxidation of the reduced insulin we obtained again a disulfide linkage but as we have stated it was inactive.   Both of the investigators mentioned have shown conclusively that insulin can be destroyed without apparently affecting the disulfide linkage.   *But, so far no one has split out or changed the disulfide grouping without destroying the potency.* Reasoning on the basis of such evidence that the disulfide has nothing to do with the activity would be like reasoning that the phenolic groups of epinephrine have nothing to do with its physiological activity because one could destroy the activity of epinephrine by masking or removing the amine grouping without touching the phenolic groups.

The only reaction which so far seems to offer the possibility of being characteristic or peculiar to insulin is the formation of the heat precipitate and its behavior.   In all the cases we have so far studied in which the insulin has been inactivated by reduction the inactivated material in the disulfide form again failed to yield the heat precipitate.   It is further evidence that the inactivated reoxidized material is fundamentally changed.   These findings with respect to the heat precipitate are very suggestive that the groups involved in the physiological action might be those necessary as well for the formation of the heat precipitate.   Further investigations are therefore under way to see whether loss in physiological activity is always associated with a loss in ability to yield the heat precipitate.

Whether or not a relationship exists between the sulfhydryl compounds of the body such as glutathione and insulin only further work can disclose.   The results obtained in the present investigation could only be interpreted as suggestive of this and there is therefore no basis as yet to assume that such a relationship exists in the animal body.

SUMMARY

1. Crystalline insulin is completely inactivated by cysteine and by glutathione.   The conditions for this inactivation have been determined.

2. Insulin is soluble in dry liquid acetamide and in liquid

ammonia.   The inactivated material is practically insoluble in dry liquid acetamide.

3. Attempts to reactivate the inactivated material by reoxidation and by alkali treatment have been uniformly negative.

4. The insulin inactivated by the above reducing agents fails to yield the heat precipitate which is so characteristic of insulin.

5. The evidence points towards the fact that the reduction of the disulfide grouping is the cause of the inactivation and that the sulfhydryl form of insulin is inactive.

BIBLIOGRAPHY

1. du Vigneaud, V., Jensen, H., and Wintersteiner, O., *J. Pharmacol. and Exp. Therap.*, **32**, 367 (1928).
2. du Vigneaud, V., *J. Biol. Chem.*, **75**, 393 (1927).
3. Mirsky, A. E., and Anson, M. L., *Proc. Soc. Exp. Biol. and Med.*, **28**, 170 (1930).
4. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **137**, 92 (1923).
5. du Vigneaud, V., Audrieth, L., and Loring, H., *J. Am. Chem. Soc.*, **52**, 4500 (1930).
6. Mason, H. L., *J. Biol. Chem.*, **86**, 623 (1930).
7. du Vigneaud, V., Geiling, E. M. K., and Eddy, C. A., *J. Pharmacol. and Exp. Therap.*, **33**, 497 (1928).
8. Blatherwick, N. R., Bischoff, F., Maxwell, L. C., Berger, J., and Sahyun, M., *J. Biol. Chem.*, **72**, 57 (1927).
9. Freudenberg, K., Dirscherl, W., and Eyer, H., *Z. physiol. Chem.*, **187**, 89 (1930).

THE RESOLUTION OF INACTIVE CYSTINE AND ISOLATION OF PURE DEXTROROTATORY CYSTINE

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The question of the identity of inactive cystine has been the subject of many investigations ever since its preparation by Mörner (1) in 1899. The literature concerned with the optical aspects of cystine has been well reviewed in recent years by Gortner and Hoffman (2, 3) and by Kahn and Goodridge (4). Furthermore in the preliminary report (5) of this work a few of the papers more pertinent to the present investigation were mentioned briefly. A detailed review of the literature therefore seems to be unnecessary at this time.

Due to the symmetry of the cystine molecule, the presence of two asymmetric carbon atoms makes possible a meso as well as a racemic modification of inactive cystine.

| | Racemic | Meso |
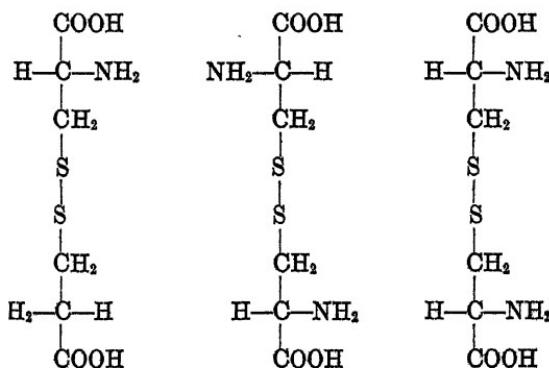
* The experimental data in this paper are taken from a thesis submitted by Leonore Hollander in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

In the heat racemization of *l*-cystine with concentrated hydrochloric acid, it seems reasonable to expect that a mixture of these two forms should result. Some workers however have suggested that inactive cystine was the internally compensated meso form (3), while others again believed it to be a mixture of meso and racemic (6). Efforts to resolve the material have hitherto met with failure (3). No positive evidence has as yet been brought forth demonstrating the presence of either the racemic or the meso form. To prove the presence of the racemic form only actual resolution with isolation of the enantiomorphs can suffice. Acceptable proof of the presence of the meso form is more complicated. Simply a lack of success in attempted resolution is not sufficient evidence. The actual isolation of mesocystine and demonstration of its failure to resolve under conditions exactly the same as those that will bring about the resolution of racemic cystine are necessary to prove the presence of mesocystine. It will be seen that conclusive proof for the characterization of mesocystine is therefore dependent on the successful resolution of racemic cystine.

The attempt to resolve inactive cystine was also undertaken in the hope of isolating *d*-cystine in order to study its chemical and physiological behavior. The isolation of *d*-cystine would make possible a study as to whether it would replace *l*-cystine in the diet. This would be particularly interesting since *l*-cystine is an essential amino acid. Furthermore a study of its catabolic fate in the animal body would also be very interesting, whether or not it were utilized for growth purposes.

EXPERIMENTAL

The inactive cystine was prepared by a slight modification of the method of Hoffman and Gortner (2). The *l*-cystine was refluxed with 12 volumes of 20 per cent hydrochloric acid for 96 hours in a sand bath on a hot plate. The solution was decolorized with norit and evaporated under diminished pressure to get rid of the greater portion of the excess hydrochloric acid. Water was then added and the solution made alkaline to Congo red but acid to litmus by the addition of sodium carbonate. In 1 per cent solution in 1 N hydrochloric acid the precipitated cystine showed no noticeable rotation. A 4 per cent solution, however, gave a very slight

residual levorotation. The yield of inactive cystine varied from 70 to 80 per cent of the theoretical.

Brucine Salt of Diacetyl-l-Cystine

In a 1500 cc. beaker packed in ice and equipped with a mechanical stirrer, 25 gm. of cystine were dissolved in 105 cc. of 2 N NaOH. From burettes 500 cc. of 2 N NaOH and 50 cc. of acetic anhydride were added in portions over a period of about 30 minutes. The solution was allowed to stand at room temperature for about 30 minutes longer and an amount of 6 N sulfuric acid added corresponding exactly to the total amount of sodium hydroxide that had been used. The solution was then evaporated to dryness under diminished pressure.

Although acetylcystine is practically insoluble in absolute acetone it is quite soluble in watery acetone. This latter solvent was used for the extraction of acetylcystine from the evaporated reaction mixture. If too much water is present, some sodium sulfate will be dissolved. In this case absolute acetone can be added to throw out the sodium sulfate. The acetone method of extraction in spite of requiring a little more care is preferable to ethyl alcohol extraction because the latter usually results in some esterification. This will be referred to subsequently in greater detail.

The acetone extract of diacetylcystine was then evaporated under diminished pressure. Absolute acetone was added and distilled, the process being repeated a number of times to drive off the water. Towards the end of the treatment the thick viscous syrup fluffs up in the flask, finally solidifying, and can be broken up and removed from the flask. Macroscopically the material appears crystalline but under the microscope no definite crystalline structure has been observed. Since the material is not definitely crystalline, it seems preferable not to introduce the melting point and rotation into the literature. The crystalline brucine salt and the crystalline ethyl ester served to characterize the compound. The free diacetylcystine is exceedingly soluble in water and alcohol, but insoluble in acetone, chloroform, ether, and ethyl acetate. The yield of the product was about 90 per cent of the theoretical amount.

To 4.5 gm. of diacetyl-l-cystine in 15 cc. of water, powdered brucine was added in small portions until the solution was neutral to lit-

mus. During the addition the solution was slightly warmed.
After the solution was cooled and allowed to stand for a few hours,
9 gm. of crystals were obtained having a rotation of $-62°$ and
melting at 147–150°. Upon recrystallization from water large
rectangular prisms were obtained. The salt, crystallized to con-
stant rotation and dried at 78° and 18 mm. pressure, had a melting
point of 148–150° (corrected) and a rotation of $[\alpha]_D^{27} = -66°$ for a
1 per cent solution in water.

Analysis
    0.1715 gm. substance: 0.0724 BaSO₄.

0.2107	"	"	: 0.0893	"			
		$C_{56}H_{64}O_8N_4S_2$.	Calculated.	S	5.94		
			Found.	"	5.80		
				"	5.82		

The free cystine was obtained after hydrolysis of the brucine salt
according to the general directions given below but 1 N acid was
used. 0.5 gm. of the typical hexagonal crystals of *l*-cystine was
obtained from 3 gm. of the brucine salt of diacetyl-*l*-cystine. The
l-cystine had a rotation of $[\alpha]_D^{26} = -212°$ in 1 per cent solution in 1
N HCl. This rotation is the maximum we have been able to ob-
tain for *l*-cystine.

Ethyl Esters of Diacetyl-l-Cystine and Diacetyl-i-Cystine

As mentioned above alcohol extraction tends towards the forma-
tion of ester. A slight trace of free sulfuric acid increases the forma-
tion to a high degree and if alcohol extraction is resorted to it is
particularly important in neutralizing the sodium hydroxide to be
very careful that there is no excess of sulfuric acid. In one of our
early experiments sulfuric acid was added until the solution was
acid to Congo red and there was a slight excess of sulfuric acid
present. On recrystallization from water of the material after
evaporation of the alcohol, 8 gm. of long needles were obtained,
melting at 122°. Further recrystallization gave a product melting
at 124.5° (corrected) and having a rotation of $[\alpha]_D^{28} = -96°$ in
2.7 per cent alcohol solution. The compound was identified as the
ethyl ester of diacetyl-*l*-cystine. The properties agreed with those
given by Cherbuliez and Plattner (7) for diacetyl-*l*-cystine pre-
pared by another method in which the cystine was acetylated after
esterification.

Analysis

    3.145 mg. substance: 0.210 cc. N at 28° and 746 mm.
    0.2100 gm.   "   : 0.2622 gm. $BaSO_4$ (Benedict).
                 $C_{14}H_{24}O_6N_2S_2$. Calculated. N 7.37, S 16.86
                          Found.   " 7.43, " 17.10

A similar product was obtained from inactive diacetylcystine. This compound also crystallized in needles from water and had a melting point of 122–123° (corrected). The rotation was zero. A mixture of the inactive ester and the levo ester gave a melting point of 116°.

Analysis

    0.1533 gm. substance: 0.0140 gm. NH_3 (Kjeldahl).
    0.1775   "   "   : 0.2167 " $BaSO_4$ (Carius).
                 $C_{14}H_{24}O_6N_2S_2$. Calculated. N 7.37, S 16.86
                          Found.   " 7.54, " 16.77

Brucine Salt of Inactive Diacetylcystine

Acetylation of the inactive cystine and preparation of the brucine salt were carried out as in the case of the levo isomer. A 1 per cent solution in water of the crude product obtained by evaporation of the reaction mixture gave a rotation of $[\alpha]_D^{25} = -22°$. The rotation of the levo salt and the approximate rotation of the salt of the inactive isomer gave some idea what rotation the brucine salt of the dextro isomer might be expected to have..

Twelve recrystallizations from water gave a product with a rotation of $[\alpha]_D = +17.5°$. In another run the brucine salt was recrystallized until the rotation remained constant. The resulting compound melted at 162–164° and had a specific rotation of $[\alpha]_D^{23} = 21°$ for a 1 per cent solution in water. The product was dried at 78° and 18 mm. pressure for analysis.

    5.933 mg. substance: 2.50 mg. $BaSO_4$.
                $C_{56}H_{64}O_8N_4S_2$. Calculated. S 5.94
                        Found.   " 5.79

Recrystallization of the inactive material from methyl alcohol also resulted in resolution yielding the dextro isomer. Another observation that later proved very helpful was that the levo isomer, although more soluble in water and in methyl alcohol than the

dextro, was less soluble in ethyl, propyl, and butyl alcohols. Recrystallization of the brucine salt of the inactive diacetylcystine from the latter solvents yielded the levo isomer. As will be seen this observation was utilized later to bring about the isolation of both the levo and dextro isomers with the same degree of purity from the same resolution.

Since the resolution with brucine is a very laborious one involving many many recrystallizations, other alkaloid salts of inactive diacetylcystine were tried. In our hands these proved less suitable than brucine.

Resolutions with brucine were carried out next on a much larger scale. Since the purpose of the present investigation was not only to find out whether racemic cystine was present and to isolate *d*-cystine for chemical and physiological studies but to obtain as well some idea as to the amount present, it was necessary to make the resolution as complete as possible. The various residues and mother liquors were therefore exhaustively recrystallized in order to obtain as large a yield of the dextro and levo isomers as possible. Naturally the size of the crops of crystals in the various fractions and their rotation varied somewhat in the various runs. We will therefore present only briefly the important steps involved in a typical resolution and indicate the general procedure to be followed.

In such a typical resolution 100 gm. of inactive diacetylcystine were dissolved in about 300 cc. of water and brucine was added gradually with stirring until the solution was neutral to litmus. About 270 gm. of the hydrated brucine were required. The solution was then allowed to remain in the ice box for 24 hours. A thick precipitate of crystals, constituting the first crop, formed. This sample consisting of 46 gm. was recrystallized three times from methyl alcohol and four times from water. 6 gm. were finally obtained, having a rotation of $[\alpha]_D^{27} = +20°$.

The mother liquor from the first crop of 46 gm. was evaporated and a second crop of 102 gm. was obtained. To this was added the evaporated mother liquor of the first recrystallization of the 46 gm. sample and the combined product weighing 113 gm. was recrystallized six times from methyl alcohol. At each succeeding recrystallization the mother liquor of the corresponding fraction from the recrystallization of the first crop was added to it. 34 gm. of crystals having a slight positive rotation were obtained. To this

were added 11 gm. of a product having approximately the same rotation which had been obtained from the sixth recrystallization from methyl alcohol of the third crop of 80 gm. of crystals from the original mother liquor. In the recrystallization of the third crop the same procedure as indicated above was utilized by adding to succeeding fractions the evaporated mother liquors resulting from the corresponding recrystallizations of the second crop. The combined 11 and 34 gm. samples were then recrystallized six more times from water, yielding 8 more gm. of the pure dextro isomer.

The mother liquor of the third crop was quite levo and did not give further crops of crystals on evaporation. The differential solubility of the isomers in the alcohol series was then brought into play. The mother liquor was evaporated to dryness and recrystallized from butyl alcohol, 65 gm. of crystals being obtained. This was recrystallized twice from butyl alcohol and three times from ethyl alcohol, yielding 16.5 gm. of the pure levo isomer.

The mother liquors from the first six recrystallizations of the third crop were evaporated and likewise recrystallized from butyl alcohol and finally from ethyl alcohol. The uniting of mother liquors with corresponding fractions was of course resorted to as in the recrystallizations on the dextro side. The general attack of utilizing first methyl alcohol and water to remove dextro fractions and then butyl and ethyl alcohol to get out the levo fractions was employed on the remaining fractions intermediate in rotation. In this way the material was worked back and forth until the mother liquors would finally yield no further crystalline fractions. This attack not only made possible the isolation of the pure levo isomer but also actually increased the yield of the dextro because residues that would no longer yield dextro fractions would do so after they had been put through the butyl or ethyl alcohol treatment to remove levo fractions. Throughout the work rotations were taken in order to follow the course of the separation. In the first resolution rotations were taken at each step but in later resolutions rotations were determined less frequently.

From this resolution 30.5 gm. of the brucine salt of diacetyl-d-cystine having a specific rotation of $[\alpha]_D^{27} = +20°$ and 46 gm. of diacetyl-l-cystine salt with a rotation of $[\alpha]_D^{26} = -64°$ were finally obtained. Besides this, 12 gm. ($[\alpha]_D^{28} = +16°$), 19.5 gm. ($[\alpha]_D^{28} = -8°$), and 26 gm. ($[\alpha]_D^{28} = -53°$) of material were

obtained. The original mother liquor and other mother liquors that no longer would yield crystalline fractions represented about 160 gm.

Other runs gave yields approximately the same so that we feel that we have subjected the material to as exhaustive a resolution as possible. The possible nature of the material remaining in the mother liquors will be discussed later.

Hydrolysis of the Brucine Salt of Diacetyl-d-Cystine

For the recovery of cystine from the brucine salt of diacetyl-*d*-cystine the brucine was filtered after the addition of alkali to the solution at a low temperature, and the remaining traces extracted with chloroform. The neutralized solution of the free diacetyl-*d*-cystine was then evaporated to a convenient volume and hydrochloric acid added to make 8 to 10 volumes of 2 N hydrochloric acid. After refluxing for 2 hours, the solution was evaporated almost to dryness, water was added, and the solution neutralized with sodium acetate. The yield of cystine varied from 65 to 80 per cent of the theoretical. The cystine can also be isolated in almost as good a yield without removing the brucine. The brucine salt itself was hydrolyzed with 10 volumes of 2 N hydrochloric acid for 2 hours and the cooled solution made just alkaline to Congo red with sodium acetate. The precipitated cystine was filtered and washed freely with water to remove any brucine acetate.

The rotation of the cystine samples so obtained varied from + 200° to +210° at 26°. Some racemization no doubt occurred. Under less drastic conditions for hydrolysis in which yield was sacrificed to get as high a rotating product as possible, the acetyl-*d*-cystine was refluxed with 1 N hydrochloric acid for ½ hour. Cystine having a rotation of $[\alpha]_D^{26} = +212°$ was obtained.

Analysis

2.759 mg. substance: 0.283 cc. N at 23° and 744 mm.
4.972 "   "   : 9.63 mg. BaSO₄.
    $C_6H_{12}O_4N_2S_2$. Calculated. N 11.67, S 26.66
                Found.   " 11.58, " 26.60

Racemic Cystine

Exactly 200 mg. of *d*-cystine and 200 mg. of *l*-cystine were dissolved in dilute hydrochloric acid. Concentrated hydrochloric acid

was added to make an acid concentration of about 20 per cent. The hydrochloride of racemic cystine crystallized in diamond platelets and thick diamond-like prismatic crystals. Some of the diamonds seemed to have two opposite corners cut off, retaining the general diamond-like appearance yet actually having six sides. The hydrochloride was strikingly different from the hydrochlorides of either *d*- or *l*-cystine which crystallized in large prismatic needles.

<div align="center">DISCUSSION</div>

The actual isolation of the dextro and levo isomers from inactive cystine produced by racemization of *l*-cystine with boiling hydrochloric acid demonstrates conclusively the presence of the racemic modification in the inactive cystine. The method of attack could not be expected to demonstrate beyond doubt whether or not the inactive cystine consisted entirely of the racemic form. Only a quantitative separation into the *d* and *l* forms could prove this and of course in actual practice no resolution results in such complete separation. Mesocystine could therefore be present and unless it possessed some characteristic solubility it might remain undetected.

The yield of 46 gm. of the pure levo isomer demonstrated the presence of 92 gm. of racemic salt in the original 311 gm. of brucine salt, calculated on the amount of brucine used. Furthermore the 26 gm. of salt with a rotation of −53° represented about 19 gm. more of the levo isomer or 38 gm. of racemic isomer. The total amount of racemic isomer shown to be present would therefore be 130 gm. There is also no doubt of there being more of this modification in the mother liquors that were left, so that the total amount of racemic cystine must be in excess of these figures.

The nature of the material remaining in the mother liquors attracts attention because of the relatively large amount. They contain of course all the impurities and decomposition products that resulted from the prolonged treatment. There is also no doubt that more of the brucine salts of the *l*- and *d*-diacetylcystines is present. Whether or not these fractions also contain the brucine salt of mesodiacetylcystine these data are unable to lead to a decision. These fractions will be further investigated.

The differential solubility of brucine salts of the two isomers in water and methyl alcohol and in ethyl, propyl, and butyl alcohols is

worthy of notice. Recrystallization from the former group brought down the dextro fractions, while from the latter the levo fractions were obtained. The difference in this respect between methyl and ethyl alcohols is particularly surprising. The use of this solubility behavior brought about the isolation of both isomers in pure form and made possible a greater yield of both forms. Such behavior of stereoisomers might be more wide-spread than is generally recognized, and is a point worthy of some attention in investigations involving resolution.

The isolation of *d*-cystine has presented the opportunity of studying the replacement in the diet of *l*-cystine with this isomer and its fate in the animal body. Experiments along these lines are already under way in this laboratory.

SUMMARY

The diacetyl derivative of cystine has been prepared by acetylation of cystine in alkaline solution with acetic anhydride. The resolution of cystine inactivated by heat and acid was accomplished by means of the brucine salt of this derivative. The resolution led to the isolation of both enantiomorphs. Dextrorotatory cystine was obtained in hexagonal crystals having a rotation of $[\alpha]_D^{26} = +212°$.

The results partly settle the much investigated question as to the identity of inactive cystine. The presence of racemic cystine has been conclusively demonstrated. Whether or not mesocystine is present cannot be answered by these results.

The diethyl esters of levo and inactive diacetylcystine have also been prepared.

BIBLIOGRAPHY

1. Mörner, K. A. H., *Z. physiol. Chem.*, **28**, 595 (1899).
2. Hoffman, W. F., and Gortner, R. A., *J. Am. Chem. Soc.*, **44**, 341 (1922).
3. Gortner, R. A., and Hoffman, W. F., *J. Biol. Chem.*, **72**, 433 (1927).
4. Kahn, M., and Goodridge, F. G., Sulfur metabolism, Philadelphia and New York (1926).
5. du Vigneaud, V., and Hollander, L., *Proc. Soc. Exp. Biol. and Med.*, **28**, 46 (1930).
6. Andrews, J. C., and de Beer, E. J., *J. Physic. Chem.*, **32**, 1031 (1928).
7. Cherbuliez, E., and Plattner, P., *Helv. Chim. Acta*, **12**, 326 (1928).

ON THE CARBOHYDRATES OF MUSCLE*

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In this investigation an attempt was made to separate and determine quantitatively the true carbohydrates in muscle. The kinetics of the acid hydrolysis of the hexosephosphoric acid esters and of the acid hydrolysis of dog muscle filtrate are presented. In an earlier paper from this laboratory (1) a method for the deproteinization and determination of total carbohydrates other than glycogen in tissue was described. It was shown also that there was an increase in the reducing power of a protein-free muscle filtrate after acid hydrolysis as was observed by Bischoff and Long (2) and recently by Kerly (3). However, this increase may not be due to the presence of a new saccharide, since the reducing power of Embden's lactacidogen according to Meyerhof and Lohmann (4) is increased upon acid hydrolysis. Lohmann (5) has reported a trisaccharide among the products of action of muscle extract on glycogen. Barbour (6) isolated a reducing trisaccharide which is formed upon the hydrolysis of glycogen by muscle glycogenase. Whether or not this carbohydrate is a normal constituent of the resting muscle has not yet been shown.

The method previously described (1) gave a satisfactory estimate of the total reducing carbohydrates in the protein-free muscle filtrate before and after acid hydrolysis, but it included, unfortunately, reducing substances other than carbohydrates. The reducing substances other than glucose found in a protein-free filtrate of blood have recently been discussed by Benedict (7) who termed them "saccharoids." This term will be used in this paper in connection with the non-sugar reducing materials in tissue.

Salkowski (8) discovered that glucose forms a complex with copper which is completely precipitated when the solution contains an excess of alkali. Van Slyke (9) made use of this discovery and proposed the well known copper sulfate and calcium hydroxide reagents which have since been widely used for the removal of carbohydrates. In this investigation we make use of the Van Slyke reagents, with a slight modification, for the separation of carbohydrates from saccharoids and describe a procedure for the complete recovery of carbohydrates from the carbohydrate-copper sulfate complex formed.

Glucose and reducing substances were estimated by the Folin and Wu method as modified by the author (10), while phosphorus was determined by the method of Fiske and Subbarow (11).

Procedure

In studying the carbohydrates of frog muscle, freezing the muscle in liquid air is not necessary, as was pointed out in a previous investigation (1). Moreover, it was there shown that the protein-free filtrate thus obtained did not contain any glycogen since upon ultrafiltration all carbohydrates present were recovered in the ultrafiltrate. If the skeletal muscle of a warm blooded animal is to be used (dog muscle was used in this investigation), it is necessary to freeze it with liquid air to prevent glycogenolysis. The technique employed is as follows: The muscle is carefully and rapidly dissected, dropped into a mortar containing liquid air, and pulverized. The required amount is then weighed and treated with the proper amount of 10 per cent copper sulfate and 10 per cent sodium tungstate along with distilled water to bring about the desired concentration. The ratios in these experiments are 1 gm. of tissue, 1 cc. of each reagent, and 2 cc. of distilled water for a final dilution of 1:5. After thorough stirring, the mixture is allowed to stand for not less than 15 minutes, when it is either centrifuged or filtered. The clear filtrate is slightly colored blue. In employing the Van Slyke reagents for the precipitation of carbohydrates in the protein-free muscle filtrate, the following proportions were found most suitable. To 10 cc. of the muscle filtrate 0.5 cc. of 10 per cent copper sulfate and 1.5 cc. of 5 per cent calcium hydroxide are added. The mixture is well shaken and is kept either on ice or in a refrigerator for not less than 45 minutes.

It is then centrifuged and the clear supernatant liquid decanted and kept (Filtrate A). For the recovery of the true carbohydrates from the precipitate, the centrifuge tube containing the precipitate is placed in a warm water bath and enough N sulfuric acid is added to dissolve the carbohydrate-copper sulfate complex and precipitate the calcium as calcium sulfate. Usually it requires about 2 to 3 cc. of N sulfuric acid to dissolve the precipitate of 10 cc. of extract. A stirring rod is introduced to facilitate the solution of the carbohydrate complex and stirring is continued until the calcium sulfate formed is perfectly white. The content of the tube is next filtered and washed thoroughly with hot water and poured over the precipitate in the filter paper. The filtrate and washings are collected and brought to a definite volume (Filtrate B).

EXPERIMENTAL

The filtrate obtained after deproteinization is termed "extract" to distinguish it from Filtrates A and B. Filtrate A in each case is the supernatant liquid that separates out after the addition of copper sulfate and calcium hydroxide. Filtrate B is the fraction obtained by recovering the true carbohydrates from the copper sulfate complex by treatment with dilute sulfuric acid. Liquid air was used in preparing muscle extracts of Dogs 4 and 5. Every experiment was performed in duplicate in full, and the results obtained checked very closely within the experimental error. The results of this experiment are found in Table I.

It is apparent from this experiment that (a) the sum of the reducing materials in Filtrates A and B, before and after acid hydrolysis, corresponds very closely to the amounts found in the original extract. Dog 3 is excepted and will be discussed separately. (b) The total amount of free and bound phosphates in the extract was recovered completely in Filtrate B. This indicates that the hexosephosphoric acid ester of the muscle is precipitated by the Van Slyke reagents. (c) The saccharoids are found in Filtrate A only, and (d) the reducing power of Filtrates A and B was increased after acid hydrolysis.

The nature of the saccharoids in muscle tissue is believed by the author to be as complicated as that found in blood and discussed in detail by Benedict (7). The only observation made by

TABLE I

Determination of Reducing Carbohydrates, Free and Bound Phosphates, and Saccharoids before and after Acid Hydrolysis of Extracts of Dog and Frog Muscle Tissue and of Their Filtrates, Filtrates A and B

Reduction is expressed in mg. of glucose per 100 gm. of tissue.

	Before hydrolysis		After hydrolysis	
	Reducing materials	Inorganic P	Reducing materials	Inorganic P
Frog 1				
Filtrate A 1	25		80	
" B 1	35	3.5	89	7.5
Total	60	3.5	169	7.5
Original extract	60	3.6	169	7.3
Frog 2				
Filtrate A 2	30		105	
" B 2	54	2.8	86	9.1
Total	84	2.8	191	9.1
Original extract	84	2.9	195	9.1
Dog 3				
Filtrate A 3	30		101	
" B 3	103	10.2	250	26.4
Remainder in calcium sulfate			51	
Total	133	10.2	402	26.4
Original extract	135	10.3	420	26.6
Dog 4				
Filtrate A 4	31		115	
" B 4	62	5.0	86	10.0
Total	93	5.0	201	10.0
Original extract	96	5.2	203	10.3
Dog 5				
Filtrate A 5	24		63	
" B 5	40		58	
Total	64		121	
Original extract	67		125	

Tissues of Dogs 4 and 5 were frozen with liquid air.

the author in this connection, which is still under investigation, is that when Filtrate A is allowed to stand for 24 hours or more the reducing substances before hydrolysis diminish considerably

without affecting the hydrolyzable reducing materials that appear upon acid hydrolysis.

The increase in reduction after acid hydrolysis of Filtrate A is probably due either to a non-reducing saccharide or to a glucoside which is not precipitated by the Van Slyke reagents. Since all the reducing materials in the extract of frog muscle were shown in a previous investigation to be ultrafiltrable, this carbohydrate cannot be of greater complexity than a trisaccharide. It forms an osazone only after acid hydrolysis, which crystallizes easily in 5 or 10 minutes after immersing the test-tube in the boiling water

TABLE II

Kinetics of Acid Hydrolysis of Filtrate B of Frog Muscle, Showing Constancy of K in Monomolecular Equation for Hexosemonophosphoric Acid Ester

Time	Reducing carbohydrates	K_1	Inorganic P	K_2
min.	*mg. per 100 cc.*		*mg. per 100 cc.*	
0	102		10.8	
10	114	0.026	12.2	0.016
20	125	0.029	13.1	0.015
30	131	0.027	14.7	0.018
60	144	0.027	16.4	0.016
75	148	0.029	17.5	0.018
90	154		19.9	
120	154		19.9	

Values for K_1 and K_2, for the acid hydrolysis of Filtrate B of dog muscle treated with liquid air, are similar to those given above.

bath. Microscopically, the crystals formed have the appearance of those of a glucosazone.

Filtrate B of frog tissue or of dog tissue treated with liquid air does not seem to contain more than two carbohydrates, as can be seen from examination of the data presented in Table II. One of these carbohydrates is very probably a glucose which is partly diffused from the blood (Cori (12)) and partly hydrolyzed from glycogen (Sahyun (1)). Since all free and bound phosphates found in the original muscle extract are present in Filtrate B, the total amount of hexosephosphoric acid ester must be present in this filtrate. According to Lohmann (5) who studied the kinetics of the acid hydrolysis of hexosephosphoric acid esters in tissue,

the hexosediphosphate yields two constants and the hexosemono-phosphate yields one when hydrolyzed.   Recently Cori and Cori (13) reported that the ester found in rat muscle is a hexosemono-phosphate and that they were unable to find in the muscle they investigated a hexosediphosphate.   In this connection the author studied the kinetics of the hexosephosphoric acid esters in tissue. It appears that when frog muscle extract or dog muscle is treated with liquid air and extracted, the ester is a hexosemonophosphate

TABLE III

Kinetics of Acid Hydrolysis of Dog Muscle Extract at 100° in 0.5 N Sulfuric Acid

Samples were removed every 10 minutes for the determination of reducing substances and of inorganic phosphate.

Time	Reducing substances	K	Inorganic P	K
min.	*mg. per 100 gm.*		*mg. per 100 gm.*	
0	163		2.6	
10	185	0.0138	7.1	0.059
20	205	0.0143	9.1	0.053
30	228	0.0158	9.5	0.033
40	236	0.0140	9.9	0.033
50	249	0.0140	10.3	0.031
60	277	0.0190	11.0	0.031
70	297	0.0232	11.5	0.031
80	314	0.0293	12.0	0.037
90	318	0.0291	12.6	
100	320	0.0282	12.6	
110	323	0.0288	12.6	
120	330		12.6	

Samples of the extract hydrolyzed in 1 N sulfuric acid for 2 hours gave the following results: 333 mg. for the total reducing substances and 12.7 mg. for the inorganic phosphate per 100 gm. of tissue.

and possesses one constant as can be seen from the data presented in Table II.

For this experiment Filtrates B of frog muscle and dog muscle (frozen with liquid air) were concentrated and studied separately by hydrolysis in 0.5 N sulfuric acid.   K was determined accord-ing to the monomolecular equation $K = \frac{1}{t} \ln \frac{a}{a' - x}$.   The con-stancy of K for the acid hydrolysis of bound phosphate indicates

clearly that the ester is a monophosphate and the constancy of K for the hydrolyzable carbohydrate is further evidence that the increase in the reducing power of Filtrate B is due to the ester in question.

When dog tissue is not treated with liquid air, the formation of a hexosediphosphate presumably takes place as Lohmann (5) has shown from the kinetics of the acid hydrolysis of the hexosephosphoric acid ester in muscle "brei." According to Lohmann, about 70 per cent of the phosphoric acid of the hexosediphosphate splits

TABLE IV

Ultrafiltration of Dog Muscle Extract

Determination of Reducing Substances and Inorganic Phosphate before and after Ultrafiltration; Also before and after Hydrolysis of Each Fraction

The reducing substances are expressed in terms of mg. of glucose per 100 gm. of tissue; inorganic P as mg. of P per 100 gm. of tissue, with potassium monophosphate as standard.

	Before hydrolysis		After hydrolysis	
	Reducing sub-stances	Inorganic P	Reducing sub-stances	Inorganic P
Ultrafiltrate 1..........................	118	6.0	236	16.5
" 2.........................	10	2.5	35	4.5
Non-ultrafiltrable fraction.............	Trace	Trace	100	3.0
Total................................	128	8.5	371	24.0
Filtrate (before ultrafiltration)........	135	9.1	400	26.0

off during the first 10 or 15 minutes and two constants are noted. The data presented in Table III confirm his findings.

In the author's experiment, the dog muscle investigated was not treated with liquid air. It was ground and then extracted. The kinetics of the acid hydrolysis of the extract were studied in 0.5 N sulfuric acid.

In a previous investigation (1) it was shown that in ultrafiltering a protein-free frog muscle extract, all reducing and hydrolyzable carbohydrates present pass through the ultrafilter. Muscle extract of a dog (not treated with liquid air), prepared as formerly described, was subjected to ultrafiltration. The results obtained are shown in Table IV.

It appears that a considerable fraction of the hydrolyzable carbohydrates was retained by the ultrafilter and did not pass through the membrane when again ultrafiltered (Ultrafiltrate 2). When the non-ultrafiltrable fraction was taken up in water, the solu-

TABLE V

Separation of Dog Muscle Carbohydrates by Alcohol

Two samples of muscle extract of two different dogs (prepared without liquid air) were treated with twice their volume of ethyl alcohol. The precipitate formed was centrifuged. The supernatant liquid was placed on a water bath to drive off the alcohol and each fraction was taken up in the same original volume. Determinations of the reducing substances and inorganic phosphate before and after acid hydrolysis are as follows. Values of the reducing substances are in terms of glucose per 100 gm. of tissue.

	Before hydrolysis		After hydrolysis	
	Reducing substances	Inorganic P	Reducing substances	Inorganic P
	mg.	*mg.*	*mg.*	*mg.*
Determination 1				
Alcoholic solution	125	Trace	210	5.3
" precipitate	15	2.6	125	7.5
Total................................	140	2.6	335	12.8
Original muscle extract	143	2.7	335	12.8
Determination 2				
Alcoholic solution	121	3.0	240	14.1
" precipitate	19	5.5	186	12.1
Total................................	140	8.5	426	26.2
Original muscle extract	139	8.9	430	26.0

On ultrafiltration of the alcoholic precipitate of dog muscle, Extract 2, the results were as follows:

	Before hydrolysis *mg.*	After hydrolysis *mg.*
Ultrafiltrate............................	21	21
Non-ultrafiltrable fraction	None	151
Total..................................		172
Before ultrafiltration...................	19	183

tion was non-opalescent but gave a reaction with iodine similar to that of glycogen and the first product of glycogen hydrolysis with acid (14). The presence of the hydrolyzable carbohydrate in the muscle extract slowed ultrafiltration considerably to about

3 cc. every 15 minutes. This rate is one-fifth that of frog muscle extract and three-fifths that of raffinose (1). Owing to the fact that it is not diffusible through a cellophane membrane No. 600, while raffinose is, its molecule must be larger than that of a trisaccharide.

When a sample of dog muscle extract (not treated with liquid air) was mixed with twice its volume of ethyl alcohol, a considerable precipitate was formed. This experiment was repeated quantitatively on the muscle extract of two different dogs (Dogs 1 and 2) and each fraction was analyzed for reducing substances and inorganic phosphates before and after acid hydrolysis. Before analyzing the supernatant alcohol solution which was removed by centrifuging, the alcohol was evaporated on a water bath and the remainder was taken up in the original volume of distilled water. The results are shown in Table V.

After dissolving in water the alcoholic precipitate of one experiment, it was ultrafiltered. All the hydrolyzable saccharide was retained in the ultrafilter, while the reducing materials passed through. This indicates that the hydrolyzable saccharide is nonreducing and the reducing materials were merely retained by it.

Finally, this polysaccharide can be precipitated with copper sulfate and calcium hydroxide (Dog 3, Table I). The author experienced considerable difficulty in attempting its recovery. Only 50 per cent of it went into solution in Filtrate B, while the other 50 per cent remained adsorbed on the calcium sulfate. In determining the adsorbed fraction, the calcium sulfate was treated with 1 N sulfuric acid, hydrolyzed, and reduction determined as glucose.

I wish to thank Dr. C. L. Alsberg for valuable suggestions and criticism.

SUMMARY

A method is described for the separation of muscle carbohydrates in frogs and in dogs. In extracts of frog muscle and of dog muscle treated with liquid air, there appears only a hexosemonophosphoric acid ester as shown from the kinetics of its acid hydrolysis. Hexosediphosphoric acid ester is formed and glycogenolysis takes place when dog muscle is extracted without the use of liquid air, as was shown from the kinetics of the acid hydrolysis of the extract.

A saccharide, which probably has a smaller molecule than a trisaccharide, is present in frog and in dog muscle extract. It is ultrafiltrable, alcohol-soluble, and not precipitated by the Van Slyke reagents. It forms an osazone only after acid hydrolysis.

When liquid air is not used in preparing dog muscle extract, a hydrolyzable, non-reducing, non-opalescent, non-ultrafiltrable, alcohol-insoluble carbohydrate is formed. It gives the glycogen color reaction with iodine and appears to be similar in character to the non-opalescent glycogen described by Sahyun and Alsberg.

BIBLIOGRAPHY

1. Sahyun, M., *J. Biol. Chem.*, **94**, 29 (1931).
2. Bischoff, F., and Long, M. L., *J. Physiol.*, **95**, 403 (1930).
3. Kerly, M., *Biochem. J.*, **25**, 671 (1931).
4. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **185**, 113 (1927).
5. Lohmann, K., *Biochem. Z.*, **194**, 306 (1928).
6. Barbour, A. D., *J. Biol. Chem.*, **85**, 29 (1929–30).
7. Benedict, S. R., *J. Biol. Chem.*, **92**, 141 (1931).
8. Salkowski, E., *Z. physiol. Chem.*, **3**, 79 (1879).
9. Van Slyke, D. D., *J. Biol. Chem.*, **32**, 455 (1917).
10. Sahyun, M., *J. Biol. Chem.*, **93**, 227 (1931).
11. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
12. Cori, C. F., *Physiol. Rev.*, **11**, 143 (1931).
13. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **92**, p. lii (1931).
14. Sahyun, M., and Alsberg, C. L., *J. Biol. Chem.*, **93**, 235 (1931).

A STUDY ON THE PHOSPHORUS DISTRIBUTION IN RAT STRIATED MUSCLE AS INFLUENCED BY AGE, DIET, AND IRRADIATED ERGOSTEROL

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The object of this study was to determine the phosphorus distribution in the gastrocnemius muscle of the albino rat as influenced by: (*a*) our normal stock diet, (*b*) age, (*c*) McCollum's Diet 3143, (*d*) McCollum's Diet 3143 plus irradiated ergosterol, and (*e*) length of time on the McCollum rachitic diet. We studied the effect of these factors on five phosphorus fractions: acid-insoluble, acid-soluble, labile plus inorganic, labile, and total phosphorus. The total phosphorus is the sum of acid-insoluble and acid-soluble phosphorus. The acid-insoluble phosphorus is the form which is not extracted by grinding with 5 per cent trichloroacetic acid and sand, followed by washing with trichloroacetic acid, and includes, of course, the phosphorus in protein and lipid combinations. The acid-soluble phosphorus represents the total phosphorus in the trichloroacetic acid extract from the muscle and includes in addition to the labile and inorganic phosphorus, lactacidogen phosphorus, pyrophosphate, adenylic acid phosphorus, and other organic phosphorus combinations. The labile plus inorganic phosphorus value as used by us was called inorganic phosphorus up to the work of Eggleton and Eggleton (1) and Fiske and Subbarow (2). The labile phosphorus is probably all phosphocreatine, as shown by the work of Fiske and Subbarow (3).

For the weaning group we used rats from two groups taken 3 weeks apart; one group from three litters and the other group from one litter. The number of experiments on each group of the same

age of the rachitic rats, the dosage of irradiated ergosterol in terms of the therapeutic dose, the number of animals both experimental and control, and the degree of rickets in the controls are given in Table I. Only one experiment was carried out on each group of animals of the same age on the stock diet. The average

TABLE I

Summary Giving Dosages and Degrees of Rickets

No. of experiments	Age	Length of experiment	Dosage of ergosterol times therapeutic dose	No. of rats		Rickets in controls
				Experimental	Control	
	days	*days*				
1	36	15	4,300	1	1	R
2	39	16	4,300	3	2	2+, 3+
3	35	13	2,800	1	1	2+
1	48	22	4,300	2	2	4+
2	49	28	4,300	2	2	4+
3	48	24	4,300	3	2	2+, 3+
4	48, 47	24	4,300	2	2	R, 3+
1	66	45	8,600	3	3	S R
2	73	48	8,600	5	5	2R, 2+, 3+
3	71	49	8,600	5	5	3S R, +, 3+
1*	87	66	4,300	1	1	4+
2*	88	67	4,300	3	3	4+
3	92–93	70	2,800	5	5	2+, 3+
4	89–92	67	30 days, 2,800 37 " 20,000	6	6	2S R, 2+ 3+
1	120±7	97	8,600	3	3	4+
1	190	169	10,000	4	4	4+

R = rickets; S R = severe rickets.
* Used only for the average of rickets with and without ergosterol.

minimum and maximum values for each type of phosphorus for each group of animals are given in Tables II and III.

Methods

The stock rats were fed our stock diet consisting of milk, sunflower seed, and a mixture of 25 per cent powdered milk and 75

TABLE II

Effect of Age on Distribution of Phosphorus in Mg. per 100 Gm. of Muscle

Age	Diet	Length of experiment	Labile + inorganic P				Labile P			
			No. of rats	Mini-mum	Maxi-mum	Aver-age	No. of rats	Mini-mum	Maxi-mum	Aver-age
days		*days*								
21	Just weaned		10	72	101	84	8	41	58	49
35–39	R	13–16	4	106	109	107	3	63	76	71
35–39	R + E	13–16	5	102	106	104	5	64	81	73
35–39	R (all)	13–16	9	102	109	105	8	63	81	73
35	S	14	4	103	116	107	3	64	82	77
47–49	R	22–28	8	85	130	110	3	58	79	70
47–49	R + E	22–28	9	103	129	110	6	57	82	73
47–49	R (all)	22–28	17	85	130	110	9	57	82	72
66–73	R	45–49	14	89	149	117				
66–73	R + E	45–49	14	100	140	119				
66–73	R (all)	45–49	28	89	149	118				
75	S	54	4	108	117	113	3	62	83	75
87–95	R	66–70	12	125	159	132	8	59	86	72
87–95	R + E	66–70	12	124	159	134	7	68	87	74
87–95	R (all)	66–70	31	133	159	133	15	59	87	73
93	S	71	5	107	122	116	4	62	71	66
120±7	R	97	3	108	120	116	3	82	85	83
120±7	R + E	97	3	112	123	118	3	60	79	71
120±7	R (all)	97	6	108	123	117	6	60	85	77
120±14	S	99±14	4	90	105	99	4	59	89	75
190	R	159	3	111	118	116	4	66	88	76
190	R + E	159	3	106	126	116	2	79	90	84
190	R (all)	159	6	106	126	116	6	66	90	79
168	S	147	4	92	97	95	2	70	74	72

S = stock diet; R = rachitic diet; R + E = rachitic diet plus irradiated ergosterol; R (all) = rachitic diet with and without ergosterol.

per cent ground yellow corn daily, and in addition, twice a week, a bread and meat mixture and lettuce or carrots. The experimental diet, with and without irradiated ergosterol, consisted of the

TABLE III

Effect of Age on Distribution of Phosphorus in Mg. per 100 Gm. of Muscle

Age	Diet	Length of experiment	Acid-insoluble P				Acid-soluble P				Acid-insoluble plus acid-soluble P			
			No. of rats	Minimum	Maximum	Average	No. of rats	Minimum	Maximum	Average	No. of rats	Minimum	Maximum	Average
days		*days*												
21	Just weaned		9	81	93	89	9	125	159	140	8	214	239	225
35–39	R	13–16	3	46	68	54	4	142	171	159	3	187	238	210
35–39	R + E	13–16	4	45	52	48	5	150	172	157	4	198	216	206
35–39	R (all)	13–16	7	45	68	51	9	142	172	157	7	187	238	208
35	S	14	4	71	79	76	4	169	189	179	4	247	266	255
47–49	R	22–28	5	55	68	61	8	150	178	162	5	205	237	222
47–49	R + E	22–28	6	42	61	52	9	153	186	163	8	194	244	213
47–49	R (all)	22–28	13	36	68	52	17	150	186	162	13	194	244	217
66–73	R	45–49	9	21	39	29	13	144	180	163	9	170	213	194
66–73	R + E	45–49	10	21	35	26	15	153	183	167	10	182	207	195
66–73	R (all)	45–49	19	21	39	28	28	144	183	165	19	170	213	195
75	S	54	3	51	64	56	4	168	186	177	3	210	243	227
87–95	R	66–70	11	27	45	34	12	162	205	180	10	193	243	216
87–95	R + E	66–70	11	29	41	32	12	171	210	182	10	200	240	214
87–95	R (all)	66–70	29	26	45	33	30	158	210	179	25	193	243	213
93	S	71	4	50	53	52	5	161	175	166	4	213	227	220
120±7	R	97	3	45	53	49	3	152	184	169	2	201	225	213
120±7	R + E	97	3	46	50	49	3	170	194	179	2	225	239	231
120±7	R (all)	97	6	45	53	49	6	152	184	174	4	201	239	222
120±14	S	99±14	3	51	56	53	4	161	180	171	3	213	233	226
190	R	159	3	48	52	50	4	169	183	177	3	217	233	225
190	R + E	159	3	50	54	52	4	166	180	173	2	216	233	224
190	R (all)	159	6	48	54	51	8	166	183	175	5	216	233	225
168	S	147	4	49	56	52	4	155	164	160	4	212	213	212

S = stock diet; R = rachitic diet; R + E = rachitic diet plus irradiated ergosterol; R (all) = rachitic diet with and without ergosterol.

McCollum Diet 3143.[1] The ergosterol-treated animals were given daily, by medicine dropper, the peanut oil containing the irradiated ergosterol, in doses representing 2800 to 20,000 rat units. We used both males and females on the rachitic diet with a predominance of males. All the normal animals older than the weaning age were males.

The gastrocnemius muscles were obtained from the rat under barbital-ether anesthesia. The gastrocnemius was dissected free from the surrounding tissues, a thread passed under the tendon of Achilles and tied tightly, and the tendon cut distal to the thread. The other end of the thread was tied to a staple in the end of a 15 inch stick. If the two muscles were to be analyzed together they were both dissected out one after the other and tied to two separate sticks. If they were to be analyzed separately, as in a few of our earlier experiments, one muscle was dissected out, frozen, and weighed, etc. before the other was dissected out.

The rat was carried into the freezing room at $-4°$. With one clip of the scissors each gastrocnemius was removed and dropped at once into liquid air. The muscles were allowed to remain in liquid air from 2 to 10 minutes, and then removed, cut from the thread, weighed to the nearest mg., placed in a chilled porcelain mortar, broken up, and ground with sand in the freezing room. Next 10 or 20 cc., depending upon the weight of the muscle, of ice-cold 5 per cent trichloroacetic acid were added while grinding. After thorough mixing, the extract was filtered through quantitative filter paper in a cold funnel resting in a 10 cc. graduated centrifuge tube specially prepared for this purpose. 2 cc. of the filtrate were collected and the funnel was transferred to another centrifuge tube and 2 cc. more of the filtrate were collected for a duplicate determination on labile phosphorus. A drop of phenolphthalein was added to each tube and 2 per cent sodium hydroxide was added, while stirring, to the end-point of phenolphthalein. Another 1 cc. filtrate was collected for total acid-soluble phosphorus.

The tubes were then removed from the freezing room and the inorganic phosphorus precipitated with the calcium mixture of Fiske and Subbarow (3). Our determinations were made accord-

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[1] McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., J. Biol. Chem., **47**, 507 (1921).

ing to Fiske and Subbarow's method except that we precipitated the excess calcium from the filtrate with the sulfuric acid necessary to develop color in their colorimetric method, removed the precipitate by centrifuging, and then used a neutral molybdate solution. The volume was made up to 25 cc. for comparison in the colorimeter. 2 cc. of the trichloroacetic acid filtrate were used for the determination of labile plus inorganic phosphorus and 1 cc. for the determination of total acid-soluble phosphorus, the volume being made up to 25 cc. in each case. The acid-insoluble phosphorus determination was run on the trichloroacetic acid precipitate with the filter paper and the sand, after washing in the cold with a 5 per cent trichloroacetic acid solution until free from inorganic phosphate. The final volume for the colorimetric determination was 50 cc. or 100 cc. depending upon the weight of the muscle.

Ashing for the total acid-soluble and acid-insoluble fractions was carried out with either sulfuric acid and nitric acid or sulfuric acid and phosphorus-free hydrogen peroxide. The acid-insoluble phosphorus determinations were never performed in duplicate because only one sample was available. The determinations on the filtrate were run in duplicate except when the two gastrocnemii were analyzed separately and on the rats just weaned. When the two gastrocnemii were analyzed separately, the determinations from the two muscles were taken as duplicates. There was not enough material from the rats just weaned to make duplicate determinations on anything except labile phosphorus. All phosphorus determinations were carried out by the colorimetric method of Fiske and Subbarow (4). The calculations for the phosphorus fractions in the acid-soluble portion were based on the assumption that the total volume of the solution was equal to the trichloroacetic acid added plus three-fourths the weight of the muscle.

DISCUSSION

The labile phosphorus varied considerably among rats of the same group, and it did not seem to be any more variable among rats of different ages than among those of the same age if we except the weaning value. The labile phosphorus within the limits of error of our methods was not influenced by the rachitic diet either

with or without ergosterol. It is the only fraction which was the same throughout for rats on both normal and rachitic diets. As a basis for an explanation of the low weaning value as compared with the higher values at all later ages, natural activity of the animal suggests itself in view of the very striking creatinephosphate increase which Ferdmann and Feinschmidt (5) found by training muscles. At weaning, the animals had not used their hind legs much nor for very long, and the creatinephosphate was low; however, within 2 more weeks the animals became very active and the creatinephosphate probably reached the maximum and constant values.

As for the labile plus inorganic phosphorus, there were no differences between the curves for the animals on the rachitic diet only and those on the rachitic diet with irradiated ergosterol. The values for labile plus inorganic phosphorus for the normal animals after 70 days of age, were below those for animals of the same age on the rachitic diet, which is the reverse of the observations of Hentschel and Zoeller (6). They obtained lower values for this fraction in rats kept for 2 to 8 weeks on the rachitic diet; otherwise their values ran about the same as ours. Thus, their highest values came at about 12 or 13 weeks of age, which was the oldest group they used, and our highest values came at about the same age. They do not state what their stock diet was, so that we cannot tell whether the difference was due to a variance in stock diets or to some other cause. Usuelli (7), working on a similar fraction in ox striated muscle, found a continuous increase from the 3 cm. fetus to the adult ox, calculated on the basis of moist weight, and scarcely any change calculated on the basis of dry weight.

The acid-soluble phosphorus was not affected by irradiated ergosterol. In the 2 weeks following weaning, there was a rapid rise in both rachitic and normal groups, but the rise in the normal was about twice as great as the rise in the rachitic groups. The highest level for the stock animals was reached by the end of 2 weeks after weaning, while the animals on the rachitic diet did not reach this level until they were 3 months of age and by that time the normal rats showed a falling off and their final level was below that of the rachitic rats.

Probably our most interesting fraction from the standpoint of

changes affected by age is the acid-insoluble phosphorus. This
fraction had its highest value at the weaning age, which is the age
for which Sinclair (8) showed the highest phosphatide content of
the whole rat. The value decreased fairly rapidly in both groups
of animals, reaching its lowest values at $2\frac{1}{2}$ to 3 months of age,
but a much lower level in the rachitic than in the normal animals.
By 4 months of age both groups showed the same values as those
reached by the stock rats at 3 months of age. In the rats up to 3
months there seemed to be a slight difference in this fraction
between those receiving irradiated ergosterol and those not. The
difference produced by the ergosterol, if there was any, made the
values differ still more from the normal. These results suggest
that the irradiated ergosterol simply hastened the transfer of
muscle phosphorus to bone formation at the expense of this type
of muscle phosphorus. However, the quantitative handling of
this fraction was so difficult that the differences may not be
significant.

No trend can be shown indicating any difference in the total
phosphorus of the muscle of the rats receiving irradiated ergosterol
and those not receiving it. Since our total phosphorus is the sum
of the two preceding fractions, it is obvious that the stock animals
would show a rise in total phosphorus immediately after weaning,
while the rats on the rachitic diet would show a decrease. A
difference in the same direction between the two groups of rats was
maintained up to 3 or 4 months of age and after that it appeared
to be in the opposite direction. Our values were lower than those
of Hentschel and Zoeller (6), which did not seem to show any
significant differences between the normals and the rats on the
rachitic diet.

Only one of the phosphorus values maintained a difference in
the same direction throughout the whole experiment between the
animals on the stock diet and those on the rachitic diet, and that
is the inorganic plus labile phosphorus. The differences exhibited
in the other fractions in the early part of the feeding experiments
had disappeared before the age of 4 months or had actually gone
in the opposite direction. These differences are what we should
expect if the variety in the two diets lay in the availability of
phosphorus to the rat. We have no direct proof, however, that
this was the cause because the stock diet varied from the rachitic

diet in other factors than phosphorus content and calcium to phosphorus ratio. We can say, however, that we believe with confidence that the difference between the effects of rachitic and stock diets on muscle phosphorus was not in the content of vitamin D.

CONCLUSIONS

A comparative study on the distribution of various forms of phosphorus in the gastrocnemius muscles of the albino rat on the stock diet and on the rachitic diet with and without viosterol leads to the following conclusions:

1. A rachitic diet causes a marked fall in the total, acid-insoluble, and acid-soluble phosphorus content.

2. The total phosphorus on the rachitic diet following weaning falls to a very low value in 50 days and then rises to a constant normal adult level; while on the stock diet there is an initial rise and then a gradual fall to the adult level.

3. The acid-insoluble phosphorus content is highest at weaning and then falls more rapidly and to a lower level in the rachitic animals, but in 90 to 100 days rises to the normal adult level.

4. The acid-soluble phosphorus value is lowest at weaning and rises more rapidly on the stock diet than on the rachitic diet. After 60 days the values are essentially the same on the two diets.

5. The labile plus inorganic phosphorus content is lower on the stock diet than on the rachitic diet after the first 5 weeks following weaning.

6. The labile phosphorus content rises abruptly in 2 weeks following weaning to normal adult values, even on the rachitic diet.

7. Irradiated ergosterol in doses from 2800 to 20,000 times the therapeutic dose did not alter the values on the rachitic diet.

BIBLIOGRAPHY

1. Eggleton, P., and Eggleton, G. P., *Biochem. J.*, **21**, 190 (1927).
2. Fiske, C. H., and Subbarow, Y., *Science*, **65**, 401 (1927).
3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
5. Ferdmann, D., and Feinschmidt, O., *Z. physiol. Chem.*, **183**, 261 (1928).
6. Hentschel, H., and Zoeller, E., *Z. Kinderheilk.*, **44**, 146 (1927).
7. Usuelli, F., *Arch. fisiol.*, **26**, 15 (1928).
8. Sinclair, R. G., *J. Biol. Chem.*, **88**, 575 (1930).

A GASOMETRIC METHOD FOR THE DETERMINATION OF LACTIC ACID IN THE BLOOD

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Method

Whole blood or serum is first treated by the tungstic acid method of Folin and Wu for the removal of protein. The filtrate is treated with $CuSO_4$ and $Ca(OH)_2$ for the removal of sugar and other interfering substances. The lactic acid in the filtrate is then oxidized to CO_2 by potassium permanganate in the Van Slyke manometric gas apparatus (1), 1 mol of lactic acid yielding 1 mol of CO_2.

The procedure is as follows: To 1 cc. of blood or serum in a 15 cc. centrifuge tube, are added 1 cc. of 10 per cent sodium tungstate and 8 cc. of $N/12$ H_2SO_4. The mixture is stirred with a glass rod at intervals for 15 minutes (5 minutes for serum) and centrifuged. 7 cc. of the supernatant liquid are removed to a second 15 cc. centrifuge tube and 1 cc. each of 14 per cent $CuSO_4$ and 14 per cent $Ca(OH)_2$ suspension are added. After agitating at intervals for 30 minutes, the tube is centrifuged and 6 cc. are pipetted into a third tube. This is acidified with 1 drop (approximately 0.04 cc.) of 10 N H_2SO_4 and 1 hour is allowed for Ca tungstate to precipitate. When more than 1 cc. of blood is available, the analysis is carried out as described with appropriate multiples of the quantities of reagents given above.

5 cc. of this solution are then introduced into the Van Slyke extraction chamber, followed by 1 cc. of 10 N H_2SO_4. The stopcock is sealed with Hg and the dissolved gases are removed by shaking 1 minute with the Hg at the 50 cc. mark. These gases are expelled and the procedure repeated, the solution being entirely returned to the extraction chamber each time. 1 cc. of

the $KMnO_4$ solution[1] is now added and the solution shaken slowly
with the Hg just below the 50 cc. mark, for 4 minutes; the shaking
is halted at the end of 2 minutes long enough to run the solution
up to the top of the tube and back, in order to wash down the sides.

The pressure of the gases produced, P_1, is read at the 0.5 cc.
mark and the solution again lowered and shaken for 1 minute.
A second reading is then made and if this fails to check, the 1
minute shakings are repeated until a constant reading is obtained.
This usually occurs after one or two trials. The temperature, t,
is now read with an accuracy of 0.1 of a degree.

The solution is allowed to go to the top of the chamber and 3
cc. of 5 N NaOH are admitted, after which the water meniscus is
lowered a short distance below the 2 cc. mark and allowed to
stand 1 minute for drainage. The pressure of the remaining
gases, P_2, is now read at 0.5 cc. The difference between P_1
and P_2 represents the pressure of CO_2 liberated from the sample
and reagents. A blank determination is carried out as described
above, save that 1 cc. of distilled water is substituted for the blood
or serum. This blank is designated as c and includes the correc-
tion for the CO_2 liberated from the reagents and the change in
manometric reading produced by the addition of the 3 cc. of
alkali.

$(P_1 - P_2 - c) \times f = $ mM lactic acid per liter[2] or
$(P_1 - P_2 - c) \times f \times 9 = $ mg. lactic acid per 100 cc. where $f = $ con-
version factor.

Calculation of Conversion Factor—The factor for converting
pressure of CO_2 to mM of lactic acid per liter is calculated accord-
ing to Equation 6 of Van Slyke and Sendroy (2):

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[1] The $KMnO_4$ solution is made as follows: To 100 cc. of 0.1 N $KMnO_4$
are added 10 cc. of 1 N H_2SO_4. The CO_2 in the solution is removed by
evacuation. It is then resaturated with air by shaking.

[2] It may be pointed out that by expressing both blood sugar and lac-
tic acid concentrations in terms of mM per liter rather than as mg. per
100 cc. the quantitative relation between these blood constituents becomes
clearer. For example, a change in blood sugar from 108 to 90 mg. per 100
cc. and a corresponding change in lactic acid from 18 to 36 mg. per 100 cc.
would be expressed in terms of mM per liter as a change in blood sugar from
6.0 to 5.0 and in lactic acid from 2.0 to 4.0. When this is expressed in the
latter units, it is seen at once that there has been an increase of 2 mM of
lactic acid and a decrease of 1 mM of sugar.

$$\text{m}_\text{M}\ CO_2\ \text{per liter} = P \times f$$

$$P \times \frac{0.05911\ i\ a}{\text{Cc. sample}} \times \frac{1}{1 + 0.00384\ t} \times \left(1 + \frac{S}{A - S}\ \alpha'\right)$$

where $P = P_1 - P_2$

$i\ =\ 1.037$

$a\ =\ 0.5\ \text{cc.}$

$S\ =\ 7.0\ \text{"}$

$A\ =\ 50.0\ \text{"}$

$\alpha' = \alpha \times \dfrac{T}{273}$

The "cc. of sample" is seen from the dilutions used in the successive precipitations to be $\frac{1}{10} \times \frac{7}{9} \times \frac{6\ 0\ 0}{6\ 0\ 4} \times 5 = 0.3863$. The conversion factors, f, for the temperatures from 15–30° are as follows:

Temperature °C	Factor	Temperature °C	Factor
30	0.0797	22	0.0838
29	0.0801	21	0.0844
28	0.0805	20	0.0850
27	0.0810	19	0.0856
26	0.0815	18	0.0862
25	0.0820	17	0.0868
24	0.0826	16	0.0875
23	0.0832	15	0.0882

Analytical Results—To test the accuracy of the method, determinations were made on pure lithium lactate, twice recrystallized, dried to constant weight, and dissolved in redistilled water. Table I gives the results. It is seen that reasonably accurate determinations can be carried out on samples of lactic acid varying in amount from 0.045 to 0.720 mg., with 0.1 to 0.2 mg. as the optimum. Known amounts of lithium lactate were also added to blood serum which was then subjected to the tungstic acid and $CuSO_4$-$Ca(OH)_2$ treatment. These results are given in Table II. The return was within 2 per cent of the theoretical.

Parallel determinations by the gasometric method and the Friedemann and Kendall (3) modification of the distillation method of Friedemann, Cotonio, and Shaffer (4) were made on (1) standard

Determination of Lactic Acid

lithium lactate solutions, (2) whole blood, (3) serum, and (4) serum to which varying amounts of lithium lactate had been added. The results of these experiments are given in Tables III, IV, and V. It was found that although both methods gave essentially the same results on pure lithium lactate solutions, the gasometric method figures on whole blood or serum were from 0.38 to 0.71 mM per liter higher than were those by the distillation method. The average difference was 0.52, the average deviation was ±0.10, the maximum deviation was +0.19 and −0.14. The difference seems

TABLE I

Gasometric Determination of Lactic Acid on Lithium Lactate Solutions of Various Concentrations

0.5 cc. samples were used.

a	c	P_1	P_2	$P_{CO_2} =$ $P_1 - P_2$ $- c$	Temper-ature	Concentration found	Concentration calculated	Per cent of theoretical
cc.	*mm.*	*mm.*	*mm.*	*mm.*	*°C.*	*mM per l.*	*mM per l.*	
2.0	3.7	110.2	47.5	59.0	25.0	15.3	16.0	95.6
2.0	3.7	111.7	47.7	60.3	25.2	15.6	16.0	97.5
0.5	7.5	206.7	75.0	124.2	24.9	7.87	8.0	98.3
0.5	7.5	206.2	76.4	122.3	25.0	7.76	8.0	97.1
0.5	7.5	145.3	75.2	62.6	24.5	3.98	4.0	99.5
0.5	7.5	143.5	74.4	61.6	24.6	3.92	4.0	98.0
0.5	7.5	114.2	75.5	31.2	24.8	1.98	2.0	99.0
0.5	7.5	113.2	73.7	32.0	24.8	2.03	2.0	101.4
0.5	7.5	97.8	74.0	16.3	25.1	1.03	1.0	103.0
0.5	7.5	98.9	75.5	15.9	25.2	1.01	1.0	101.0
Average.								99.1

to be approximately the same for both high and low blood lactic acid concentrations. This was found to be the case for variations occurring in blood as drawn (Table IV) and in blood whose lactic acid concentration had been increased by the addition of known amounts of lithium lactate (Table V).

In view of the fact that the distillation method gives theoretical results with standard lactate solutions but with blood leads to lower results than the gasometric method, it is probable that some constituent other than lactic acid reacts with permanganate to yield CO_2. Because the difference between the two methods is

relatively constant for varying lactate concentrations, it is proposed that 0.5 mM per liter be subtracted from results obtained gasometrically. Lactate concentrations obtained in this way will be consistent with those obtained by the Friedemann, Cotonio, and Shaffer method. It is, therefore, suggested that the calcula-

TABLE II

Gasometric Determination of Lactic Acid on Serum to Which Quantities of Lithium Lactate Were Added before Tungstic Acid and Tungstic Acid Plus $CuSO_4$-$Ca(OH)_2$ Precipitations

Treatment	Lactate found		Added lactate		
	Serum	Serum plus lithium lactate	Found	Added	Per cent added lactate found
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	
Tungstic acid precipitation	7.84	15.84	8.00	8.00	100.0
	5.78	9.82	4.04	4.00	101.1
	6.24	10.16	3.92	4.00	98.0
Tungstic acid precipitation + $CuSO_4$-$Ca(OH)_2$ precipitation	7.63	11.66	4.03	4.00	100.9
	6.55	10.61	4.06	4.00	101.4

TABLE III

Parallel Determinations of Lactic Acid by Gasometric and Distillation Methods after Tungstic Acid and $CuSO_4$-$Ca(OH)_2$ Precipitations

Solution No.	Gasometric	Distillation	Difference
	mM per l.	*mM per l.*	*mM per l.*
1	7.86	7.93	−0.04
2	4.08	3.95	+0.13
3	3.91	3.88	+0.03
4	3.93	3.84	+0.09

tion of blood lactate concentrations determined gasometrically be made according to the equation:

$$\text{Blood lactate (mM per liter)} = (P_1 - P_2 - c) \times f - 0.50$$

Of substances which might be present in blood, the following yield no carbon dioxide when treated as in this method: glycine, alanine, β-hydroxybutyric acid, and urea. Those yielding a small

amount of CO_2 but not reacting quantitatively are uric acid and dextrose.   Those found to react quantitatively with permanganate were acetoacetic acid and pyruvic acid.   Since dextrose is removed by treatment with $CuSO_4$-$Ca(OH)_2$, the high results cannot be

TABLE IV

Comparison of Lactic Acid Determinations on Serum by Gasometric and Distillation Methods

The results are expressed in mM per liter.

Blood sample	Gasometric	Distillation	Gasometric minus distillation	Gasometric minus 0.50	Blood sample	Gasometric	Distillation	Gasometric minus distillation	Gasometric minus 0.50
Dog 1	7.22	6.70	0.52	6.72	Dog 7	3.19	2.68	0.51	2.69
" 2	9.88	9.22	0.66	9.38	" 8	3.50	3.11	0.39	3.00
" 3	5.73	5.26	0.47	5.23	Human 9	1.23	0.71	0.52	0.73
" 4	4.04	3.66	0.38	3.54	" 10	1.73	1.14	0.59	1.23
" 5	6.96	6.56	0.40	6.46	" 11	1.86	1.22	0.64	1.36
" 6	5.86	5.15	0.71	5.36					

Average difference...................................... 0.52

TABLE V

Comparison of the Gasometric and Distillation Methods for Determination of Lactic Acid in Serum to Which Lithium Lactate Has Been Added

The results are expressed in mM per liter.

Sample No.	Lithium lactate added	Gasometric	Distillation	Gasometric minus distillation
1-a	3.29	6.41	6.01	0.40
2-a	6.58	9.66	9.23	0.43
3-a	9.87	12.99	12.43	0.56
1-b	5.64	9.60	9.28	0.32
1-c	2.82	7.09	6.52	0.57

Average.   0.46

attributed to it.   Parallel experiments with and without the removal of acetoacetic acid failed to show any difference in results. Therefore, since pyruvic acid could hardly be expected to be present in a concentration of 0.5 mM per liter, it must be concluded

that the reason for the high yield of CO_2 by the gasometric method is unknown.

Parallel determinations on whole blood and serum showed that lactate concentrations of whole blood were approximately 80 per cent those of serum. Since lactate ions are probably diffusible and exist in lower concentration in erythrocytes than in serum, it is felt that lactate determinations should be made on serum or plasma rather than on whole blood whenever practical, in order to eliminate errors in interpretation due solely to changing proportions of red blood cells.

Special Points in Technique—0.1 per cent Na fluoride should be added to the blood on withdrawal to inhibit glycolysis, in case precipitation with the Folin-Wu reagents is not immediately carried out. After the tungstic acid and $CuSO_4$-$Ca(OH)_2$ treatment has been completed, the solutions may be safely kept in the refrigerator for periods up to 2 days with no appreciable change.

Throughout the handling of the solutions, care should be taken to avoid the introduction of any foreign oxidizable material. Cork stoppers should not be used and since a substance is obtained from even the best acid-washed filter paper which yields CO_2 with $KMnO_4$, centrifuging is used in place of filtering.

While work on the method just described was in progress Baumberger and Field (5) published a preliminary report of a method for the determination of quantities of 0.5 to 1.0 mg. of lactic acid in pure solution, through oxidation to CO_2 in the Van Slyke manometric gas apparatus by $MnSO_4$ and $KMnO_4$ in the presence of H_2SO_4. As far as we are aware no subsequent report of their method has appeared. Other recent lactic acid methods depending upon the conversion of lactic acid to acetaldehyde and its subsequent titration which have been developed to a point of considerable accuracy have been those of Jervell (6), Boyland (7), and Friedemann, Cotonio, and Shaffer.

The advantages of the gasometric method over these are the small amount of material required, the speed and simplicity of determination, and the lack of need for special apparatus other than the Van Slyke manometric gas apparatus. Its disadvantage lies in the fact that an empirical correction must be applied if results comparable to those obtained by the distillation method are desired.

We are indebted to Dr. Theodore E. Friedemann for his help in making a comparison of the gasometric and the distillation methods, and to Miss M. Eleanor Blish for assistance in the early work on the method.

BIBLIOGRAPHY

1. Van Slyke, D. D., *J. Biol. Chem.*, **73**, 121 (1927).
2. Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **73**, 127 (1927).
3. Friedemann, T. E., and Kendall, A. I., *J. Biol. Chem.*, **82**, 23 (1929).
4. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, **73**, 335 (1927).
5. Baumberger, J. P., and Field, J., Jr., *Proc. Soc. Exp. Biol. and Med.*, **25**, 87 (1927).
6. Jervell, O., *Acta. med. Scand.*, **21**, suppl. 24 (1928).
7. Boyland, E., *Biochem. J.*, **22**, 236 (1928).

THE STATE OF THE BLOOD SUGAR AS SHOWN BY COMPENSATION DIALYSIS IN VIVO*

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It has generally been accepted that the greater part of the sugar in the blood is glucose, although lactose, galactose, maltose, fructose, and pentose have been reported to be present under particular circumstances. The evidence dealing with the nature of the blood sugar has been summarized by Grevenstuk (9), who pointed out that at that time the presence of glucose had not been proved by its isolation and study in crystalline form. This deficiency has recently been supplied by the work of Winter (24). Whether sugars other than glucose are present under normal conditions is a much debated question. The precise amount of glucose present, its distribution between the cells and plasma, the manner in which it is held in the plasma, whether in a state of simple solution or partly in a colloidal or non-diffusible form, and whether other so called mutarotatory forms of glucose are present, still are disputed questions.

The investigations of Michaelis and Rona (13) with compensation dialysis *in vitro* and those of von Hess and McGuigan (12) with dialysis *in vivo* indicated that the sugar in the plasma was in a state of simple solution, but their experiments have not been accepted as being wholly definitive. Studies of the ultrafiltrate of plasma or serum obtained *in vitro* have yielded contradictory results. Rusznyák (17), Rusznyák and Hetényi (18), and de Haan and van Creveld (10) all considered that their results indicated the presence of considerable colloidal or non-filtrable sugar in the

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plasma. Delaville and Richter-Quittner (3) found the sugar content of the plasma, the ultrafiltrate, and the residue to be the same, whereas Paul (14) found that the ultrafiltrate contained slightly more sugar than the plasma. These manifold differences are probably to be ascribed to the conditions under which the different ultrafiltrates were prepared, and to the particular analytic methods used. Brull (2), applying a method of ultrafiltration *in vivo*, reported a much larger proportion of the total sugar to be present in a colloidal or non-filtrable state than was accepted by previous workers. There is a progressive fall in the concentration of sugar in the heart-lung preparation which he used. He suggested that in this preparation a point is reached at which the physicochemical state of the plasma sugar is suddenly changed to one of free and complete diffusibility.

Various studies on the closely related problem of the distribution of the sugar between the corpuscles and plasma have been reported in the case of human blood in which concentrations of sugar in the corpuscles were found to range from zero up to values corresponding with the plasma sugar. Glassmann (7) contended that the erythrocytes of human beings contain only protein sugar. Von Hess and McGuigan concluded that the corpuscles of dog blood contain no sugar and "may be considered merely as so many pebbles causing solid displacement of the plasma." In their work, anesthetics were used which are known to raise the level of the blood sugar. Other investigators have objected to the use of defibrination or of anticoagulants other than hirudin, fearing the effect of these substances on the permeability of the cells, and therefore on the apparent distribution of sugar. Somogyi (20) has pointed out the technical faults in some of the earlier experiments. To avoid possible difficulties of this nature, Stammers (22) advocated the "living test tube" method of centrifuging the blood within a ligated section of vein, and has reported some results with that method. His experiments were made under ether anesthesia, and so fail to show definitely whether the distribution of glucose *in vivo* under normal conditions is the same as that found by the analysis of drawn blood.

In the present study of these problems we have modified the technique of vivi-diffusion introduced by Abel, Rowntree, and Turner (1) to enable us to obtain a dialysate which is in equilibrium

with the circulating arterial blood under normal physiologic conditions. Such a dialysate contains the diffusible constituents of the blood in the concentrations in which they may be assumed to be available to the lymph and tissue fluids, and represents the state of diffusible substances in the blood as accurately as may be realized experimentally. If a significant amount of a non-diffusible form of reducing sugar is present, it should be detected by differences in the sugar content of the blood or plasma and the dialysate. The dialysate is protein-free and so is particularly suited to a study of the amount and properties of the blood sugar.

Methods

Dialysis was carried out *in vivo* in a dialyzing assembly similar to the small apparatus of Abel, Rowntree, and Turner, and to that of von Hess and McGuigan. This apparatus was designed to provide as large a dialyzing surface as possible, relative to the volume of the dialysate, and thus to hasten the attainment of equilibrium (Fig. 1). The dialyzing tubes were made from a 7 per cent solution of parlodion in a mixture of 3 parts of ether and 1 part of alcohol. The technique of their preparation was approximately that described by Abel, Rowntree, and Turner. The membranes were cast inside glass tubes of 5.5 to 6.5 mm. internal diameter. After evaporation of the ether, the tubes were placed in 70 per cent alcohol for 10 to 15 minutes and then transferred to distilled water. This procedure produced dialyzing membranes possessing the optimal combination of toughness, durability, and permeability. While working under water the collodion tubes were removed from the casting tubes, cut to the proper length, slipped over the glass tubes of the dialyzing apparatus, and tied in place with heavy thread. The ends of the tubes and the ligatures were then covered with successive coats of collodion solution. After drying and shrinking, this provided a tight seal at the joint, with no pockets for the accumulation or stagnation of blood. The Pyrex glass test-tube, which served as a jacket, and all connections were sterilized by autoclaving. The dialyzing tubes were sterilized by soaking in dilute alcohol. Before they were used they were rinsed with sterile Ringer's solution and then

inserted into the jacket, which was filled with a sterile Ringer's solution.

Dogs 12 to 15 kilos in weight, which had been fasted for 18 to 24 hours, were used. All operative procedures were carried out either with local anesthesia, procaine only being used, or with general anesthesia, sodium isoamylethylbarbiturate being used. The femoral artery and vein on each side were exposed and paraffin-coated glass cannulas inserted. The latter were connected to the dialyzing tubes by short pieces of paraffin-coated rubber tubing. Clotting was prevented by intravenous injection a few minutes
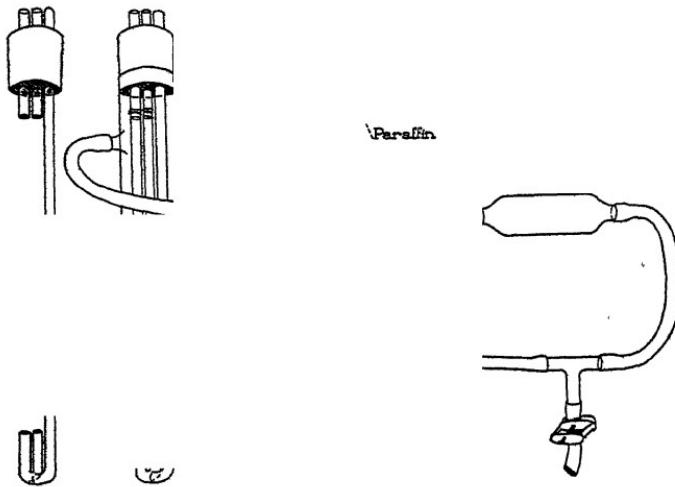
Fig. 1. Details of the dialyzing assembly. The cross-section illustrates the layer of paraffin in the outside jacket used to minimize the volume of the dialysate.

before starting the experiment of 10 mg. of heparin for each kilo of body weight. Subsequently, smaller amounts were injected as required. Under these conditions there was a rapid and efficient circulation of blood through the dialyzing tubes. A pressure approximately equal to the diastolic blood pressure was applied to the jacket of the dialyzing apparatus. This produced vigorous pulsation in the collodion tubes and served to stir the dialysate. More complete mixing of the dialysate at the time of withdrawing samples for analysis was accomplished by raising and lowering the attached bulb.

During the course of an experiment a fine deposit of clumps of platelets and of fibrin formed over the inside of the dialyzing tubes. Occasionally the deposit of fibrin was so extensive as to form a complete membrane lining the collodion tubes. In such instances the apparatus was disconnected and was washed out with Ringer's solution. If the dog was kept in good condition, and rapid and vigorous circulation was maintained, gross deposits of fibrin either were not formed or were swept away in the blood stream before coalescing into a continuous membrane.

The jackets of the dialyzing apparatus were filled with modified Ringer's solutions. The initial solution on the one side did not contain glucose (Dialysate A) but 200 mg. per cent of glucose were added to the fluid on the opposite side (Dialysate B). One solution, therefore, was hypotonic, the other hypertonic with reference to the glucose in the blood. Samples of the dialysates were analyzed at intervals and the progress toward apparent equilibrium was followed. Dialysis was usually continued for an hour thereafter to insure completeness of the equilibrium, the whole experiment lasting from 5 to 7 hours. The dialysates were completely protein-free by the usual tests. Samples of arterial blood and plasma were taken at the beginning, the end, and at intervals during the experiment to insure the constancy of the level of the blood sugar. The samples of blood were taken under oil, centrifuged immediately, and the plasma separated as rapidly as possible in order to minimize any changes *in vitro*. The samples of blood were precipitated immediately, with sodium tungstate and acid, according to the procedure of Haden (11); the plasma, according to that of Wu (26). The reducing power of the filtrates of blood and plasma and of the dialysates was determined by both the Folin-Wu (6) and the Somogyi (19) modifications of the Shaffer-Hartman methods. In the latter method potassium iodide was not incorporated in the copper reagent but was added later according to the suggestion of DeLong (4). The fermentable sugar present was determined by treating the protein-free filtrates and the diluted dialysates with washed yeast according to a slight modification of the technique of Somogyi. The analytic methods were carefully checked against pure glucose obtained from the United States Bureau of Standards, and curves prepared for each particular set of reagents were used for the calculations.

Results

The equilibrium established between the circulating blood and the dialysates is illustrated in Fig. 2, which presents the results of a typical experiment done under local anesthesia. The blood sugar was constant throughout the experiment. The plasma sugar varied more than that of the whole blood, but the changes were slight. The concentrations of sugar in the two dialysates, initially
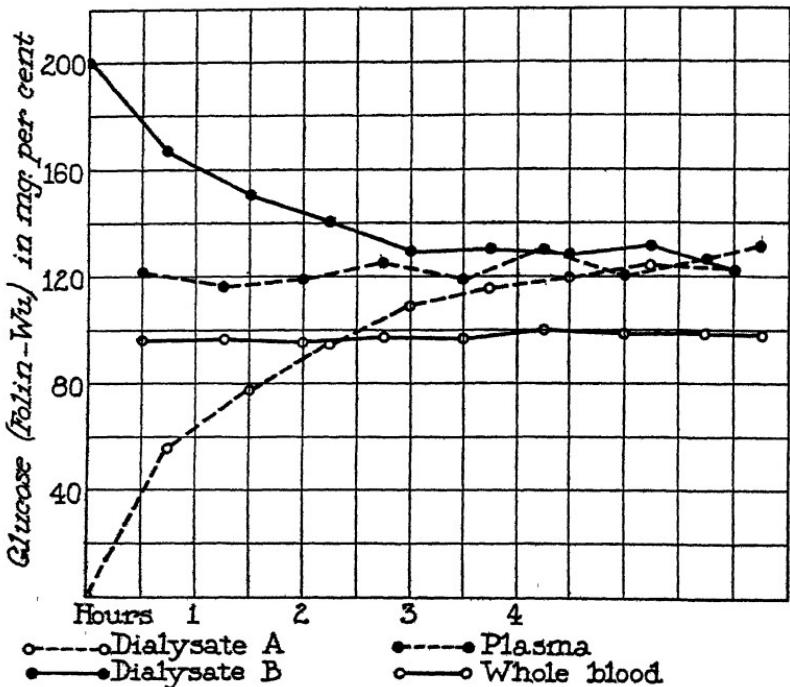
FIG. 2. Curves illustrating the equilibrium between the circulating plasma and the *in vivo* dialysates in the normal animal.

hypotonic and hypertonic respectively, progressively approached equality, and, during the last 2 hours, practically identical analytic values were obtained. This steady state coincides with the plasma sugar level and demonstrates the complete diffusibility of the reducing substances in the plasma. From the standpoint of the study of the equilibrium attained the six experiments grouped together as Series 1 in Table I were the most satisfactory in that analyses during the experiments indicated a reasonably con-

stant plasma sugar level. In these experiments the average concentration of glucose in the two dialysates at the end of each experiment was equal to, or slightly greater than, that in the plasma, the differences ranging from −3 to +14 mg. There is thus the suggestion that the concentration in the plasma water

TABLE I

Concentration of Reducing Substances in Blood, Plasma, and in Vivo Dialysates at Equilibrium

The results are expressed as mg. of glucose in each 100 cc.

Series No.	Experiment No.	Blood		Plasma		Dialysate A		Dialysate B			Anesthetic
		Folin-Wu	Shaffer-Hartman	Folin-Wu	Shaffer-Hartman	Folin-Wu	Shaffer-Hartman	Folin-Wu	Shaffer-Hartman	Average, Shaffer-Hartman	
1	4	95	107	114	119			112	125	125	Local
	6	97	110	130	128	127	130	124	130	130	"
	9	86	92	107	103	111	113	123	121	117	"
	10	106		130		137		135		136	"
	13 (total)		129		138		136			136	General
	(fermentable)		120		137		134			134	"
	15 (total)		116		133		125		138	131	"
	(fermentable)		102		128						"
2	2	140	132	160	158		158		164	161	Local
	5	94	108	115	124	99	103	128	130		"
	11 (total)		106		116		132		129	130	General
	(fermentable)		100		114		130		128	129	"
	12 (total)		132		158		130		145		"
	(fermentable)		122		148		128		143		"
	14 (total)		152		184		104		155		"
	(fermentable)		140		183		102		152		"

is the factor which determines the final equilibrium. There were, however, unavoidable fluctuations of similar magnitude in the plasma sugars so that a definite decision as to this point is difficult to make from the data at hand. Paul found a higher concentration of sugar in the ultrafiltrate than in plasma. His experiments likewise indicate that the plasma does not contain

colloidal sugar and they support the view-point that the sugar is freely dispersed in the plasma water. In Series 2, Table I, are listed those experiments in which, for one reason or another,
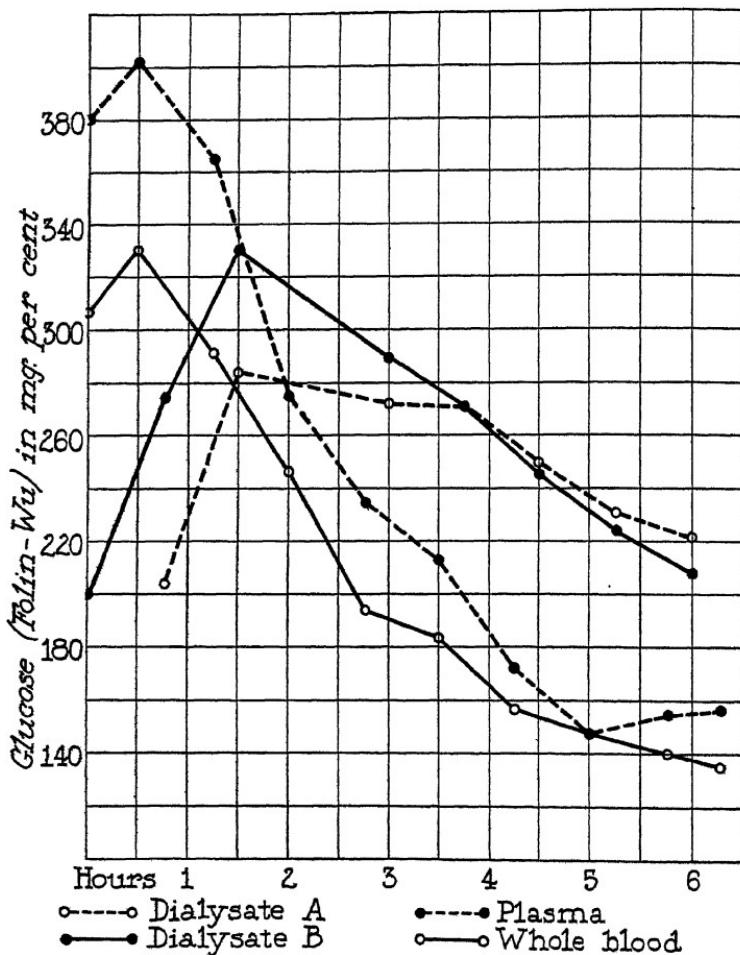
FIG. 3. Curves illustrating the relationships between the plasma sugar and dialysate sugar concentrations in an experiment with ether anesthesia.

equilibrium was delayed, or in which the value for blood sugar was too high or too variable to warrant consideration in the evaluation of the normal equilibrium.

Von Hess and McGuigan, who were the first to study the blood sugar by dialysis *in vivo*, also reported that exact equilibrium was established between the dialysate and plasma, if the concentration of glucose in the latter was expressed in terms of plasma water. We have indicated that this is probably the case, but we feel, nevertheless, that the results of their reported experiments should be accepted with some caution, for they were all carried out on animals anesthetized with morphine, morphine and ether, urethane, or urethane and ether. The constancy of the level of the blood sugar is essential to experiments such as these, but it was not established by them. The effect of these anesthetics in raising the blood sugar is well known. The effect on the sugar content of the dialysate is shown in our Experiment 7 (Fig. 3), which was carried out with ether as an anesthetic. Dialysis was started an hour after the beginning of anesthesia. At this point the value for plasma sugar was very high, 380 mg. for each 100 cc. It rose slightly in the next half hour, and then fell progressively during the rest of the experiment. The sugar content of the dialysates followed the blood sugar curve, but with a lag of between 2 and 3 hours. Equilibrium at a stationary level was never established, although a single analysis at about $1\frac{3}{4}$ hours after the beginning of dialysis would have suggested the attainment of equilibrium. Analysis at any succeeding time would have placed the equilibrium at a much higher level than that of the plasma sugar. Similar difficulties were apparently encountered by Brull in his studies of ultrafiltration *in vivo* with heart-lung preparations, in which the blood sugar falls progressively. These criticisms, we feel, do not apply to our other experiments because the constancy of the blood sugar was checked by repeated analyses. As the same steady state was realized from either side, there can be no doubt that true equilibrium between the plasma and the two dialysates was attained, and that all of the plasma sugar is in a freely diffusible condition.

The possible effect of the non-specific character of the methods of analysis of blood sugar on the interpretation of the results has been carefully considered. It is seen in Table I that the results for the total reducing substances of the whole blood filtrates, calculated as glucose, are somewhat higher by the Shaffer-Hartman method than by the Folin-Wu method. This is due to the now

generally appreciated fact that such filtrates contain considerable non-sugar, non-fermentable reducing substances which are derived principally from the cells. The various sugar reagents are affected to different extents by these substances (21), in the present instance the Folin-Wu reagent less than the Shaffer-Hartman. The non-fermentable residues in the plasma filtrates amount to only a few mg. and the plasma sugars determined by the Folin-Wu and Shaffer-Hartman methods are therefore in excellent agreement. This is even more striking in the case of the dialysates, which contain, usually, not more than 1 to 3 mg. of non-fermentable reducing material. In this respect the dialysates are slightly lower than the circulating plasma, indicating that under the conditions of the experiments the non-fermentable reducing, substances in dog plasma may be only partly diffusible. Of other, sugars besides glucose that might be included in the value for fermentable reducing substances fructose is the only one that is fermented at a rate comparable to that of glucose in the technique used. The presence of significant amounts of fructose in the plasma in these experiments can be excluded. Under these circumstances we believe that the specificity of the fermentation procedure, the very low values for the non-fermentable residues in both plasma and dialysates, and the excellent agreement of the various chemical as well as the polarimetric (16) methods of analysis on these solutions constitute evidence that the only diffusible reducing sugar present in the plasma in significant amounts is α, β-glucose.

Another question that must be considered in the interpretation of these results is the effect on the plasma sugar of shifts in the distribution of reducing substances between the cells and plasma, since it has been suggested that the distribution *in vivo* may be different from that found by the analysis of drawn blood. According to Folin and Svedberg (5) the non-fermentable reducing substances in the cells are not diffusible. The results given indicate that the only reducing substance normally present in the plasma in significant amounts is glucose and that the latter is in a freely diffusible state. Any changes due to redistribution *in vitro* would require the diffusion of glucose either into or out of the cells, and the plasma glucose as determined would be correspondingly in error. That these processes do not occur is suggested by

the relative constancy of the concentration of reducing substances in the cells. Table II shows the values obtained for the total, and in some cases the fermentable, reducing substances in the cells. These were calculated in the usual manner from the analysis of whole blood and plasma and from the hematocrit readings, and are qualitatively in accord with the similar data for dogs reported by Wishart (25) and by Trimble and Maddock (23). In the present experiments the plasma sugars are not excessively

TABLE II

Distribution of Reducing Substances between Cells and Plasma

Experiment No.	Hematocrit, per cent cells	Blood sugar	Plasma sugar	Calculated cell sugar	Cell sugar / Plasma sugar
		mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	
2	46.5	132	158	102	0.65
4	42.2	107	119	88	0.74
5	47.0	108	124	90	0.73
6	53.0	110	128	94	0.74
7	35.2	134	156	94	0.60
9	49.4	92	103	81	0.78
10	47.7	106	130	80	0.61
11	40.0	106	116	91	0.78
		100*	114*	79*	0.69*
12	32.5	132	158	77	0.49
		122*	148*	68*	0.46*
13	28.0	129	138	106	0.77
		120*	137*	77*	0.56*
14	37.7	152	184	98	0.53
		140*	183*	69*	0.38*
15	40.0	116	133	90	0.68
		102*	128*	63*	0.49*

* Fermentable reducing substances.

high, but a considerable range of concentrations is represented. Yet, whether the plasma sugar is high or low, the cell sugar is always lower and relatively constant throughout this range. Furthermore, as has been pointed out, the concentration of glucose in the dialysates at equilibrium may be taken as an index of the true concentration of glucose in the circulating plasma. This concentration was the same as that found in plasma separated *in vitro* according to the usual technique. These results thus indicate that there was no significant redistribution of glucose

between the cells and plasma of drawn blood. If glycolysis is avoided therefore, the concentration of glucose in the erythrocytes of the dog may be satisfactorily determined by the usual technique without fear of significant changes *in vitro*.

SUMMARY

A method for the compensation dialysis of the circulating arterial blood *in vivo* is described. With heparin as an anticoagulant the blood sugar has been studied in normal dogs, under either local or general anesthesia.

The reducing material in the dialysates is almost wholly fermentable; this fact together with other evidence warrants the conclusion that glucose is the only diffusible reducing sugar present in the plasma in significant amounts. The non-fermentable reducing substances in the dialysates amount to only 1 to 2 mg. for each 100 cc.

At equilibrium the concentration of glucose in the *in vivo* dialysate is equal to or slightly higher than that in the plasma. There was no evidence of the presence in the plasma of a non-diffusible or colloidally bound form of sugar. The plasma glucose is freely diffusible. Its effective concentration very likely is that expressed on the basis of the water content of the plasma. This is the concentration which determines the glucose available for diffusion into the lymph and tissue fluids.

The concentration of glucose in the erythrocytes of the dog is fairly constant over a considerable range of concentrations of plasma glucose. However, it is always less than in the plasma. There was evidence in these experiments that there is no significant redistribution of glucose between the cells and plasma in drawn blood.

BIBLIOGRAPHY

1. Abel, J. J., Rowntree, L. G., and Turner, B. B., *J. Pharmacol. and Exp. Therap.*, **5**, 275 (1914).
2. Brull, L., *Arch. internat. physiol.*, **32**, 138 (1930).
3. Delaville and Richter-Quittner, M., *Compt. rend. Soc. biol.*, **91**, 595 (1924).
4. DeLong, W. A., *J. Biol. Chem.*, **72**, 731 (1927).
5. Folin, O., and Svedberg, A., *J. Biol. Chem.*, **88**, 715 (1930).
6. Folin, O., and Wu, H., *J. Biol. Chem.*, **41**, 367 (1920).

7. Glassmann, B., *Z. physiol. Chem.*, **158**, 113 (1926).
8. Greene, C. H., and Power, M. H., *Am. J. Physiol.*, **90**, 367 (1929).
9. Grevenstuk, A., *Ergebn. Physiol.*, **28**, 1 (1929).
10. de Haan, J., and van Creveld, S., *Biochem. Z.*, **123**, 190 (1921).
11. Haden, R. L., *J. Biol. Chem.*, **56**, 469 (1923).
12. von Hess, C. L., and McGuigan, H., *J. Pharmacol. and Exp. Therap.*, **6**, 45 (1914).
13. Michaelis, L., and Rona, P., *Biochem. Z.*, **14**, 476 (1908).
14. Paul, J. R., *J. Clin. Inv.*, **3**, 631 (1927).
15. Power, M. H., and Greene, C. H., *J. Biol. Chem.*, **74**, p. xix (1927).
16. Power, M. H., and Greene, C. H., *J. Biol. Chem.*, **94**, 295 (1931).
17. Rusznyák, S., *Biochem. Z.*, **113**, 52 (1921).
18. Rusznyák, S., and Hetényi, G., *Biochem. Z.*, **121**, 125 (1921).
19. Somogyi, M., *J. Biol. Chem.*, **70**, 599 (1926).
20. Somogyi, M., *J. Biol. Chem.*, **78**, 117 (1928).
21. Somogyi, M., and Kramer, H. V., *J. Biol. Chem.*, **80**, 733 (1928).
22. Stammers, A. D., *Physiol. Rev.*, **6**, 630 (1926).
23. Trimble, H. C., and Maddock, S. J., *J. Biol. Chem.*, **78**, 323 (1928).
24. Winter, L. B., *Biochem. J.*, **24**, 851 (1930).
25. Wishart, M. B., *J. Biol. Chem.*, **44**, 563 (1920).
26. Wu, H., *J. Biol. Chem.*, **51**, 21 (1922).

THE NATURE OF THE BLOOD SUGAR AS SHOWN BY A COMPARISON OF THE OPTICAL ROTATION AND THE REDUCING POWER OF THE IN VIVO DIALYSATE*

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The results of simultaneous analyses of the blood sugar by the polarimetric and the reduction methods have rarely been found to be in satisfactory agreement. The older work has been reviewed in part by Stepp (15), together with a discussion of the possible disturbing factors, such as the presence in the blood of reducing substances or optically active substances besides glucose. In his experiments, Stepp found the optical rotation and reducing power of normal blood filtrates which had been concentrated *in vacuo* to be usually in good agreement. These values were lower than the reducing powers of the original filtrates before concentration. When filtrates from diabetic blood were concentrated there was also a loss of the reducing substances, which was explained in part by the detection of volatile reducing substances in the distillate. The optical rotation of such preparations was, however, always lower than the corresponding reducing power, due, Stepp considered, to the presence of unidentified levorotatory substances.

Other observers have assumed that the differences noted by them between the optical rotation and the reducing power of blood filtrates were significant of changes in the nature of the sugar present, and have drawn extensive deductions therefrom regarding the nature of the sugar present in normal and diabetic blood. Maase and Tachau (10) obtained a much higher value

for the blood sugar by the polarimetric than by the reduction method of analysis in a case of severe diabetes, and so assumed the presence in this instance of a sugar with a greater specific rotation but lower reducing power than ordinary glucose. Winter and Smith (20), and Lundsgaard and Holbøll (9), likewise found that in diabetes the optical rotation of blood filtrates tended to be equal to, or greater than, the reducing power. In preparations from normal blood, on the other hand, the optical rotation was low as compared to the reducing power; but on standing it gradually increased to a level corresponding to that calculated from the reduction. They interpreted these results as demonstrating the initial presence in normal blood of a labile, low rotating form of glucose. Using the same methods, Nakahayashi and Abelin (11) were able to confirm the results of Winter and Smith on normal blood. Barrenscheen, Kahler, and Hechl (3) prepared protein-free blood filtrates in a different manner, but in the main also confirmed the contentions of Winter and Smith. Thomas (17) found the optical rotations of *in vitro* dialysates of dog, cattle, rabbit, and horse blood to be lower than the corresponding reductions and likewise suggested the presence in these bloods of forms of glucose with different specific rotations than that of the ordinary α,β mixture.

The experimental evidence on which these theories of Winter and Smith, and of Lundsgaard and Holbøll, are based, has since been scrutinized with great care but with variable results, depending apparently on a number of incidental factors such as the chemical method of analysis used, the type of protein precipitant and conditions of its use, the pH of final solution, and so forth. Eadie (5), Denis and Hume (4), Thannhauser and Jenke (16), and Visscher (18), among others, on repeating the work of Winter and Smith found little if any evidence in support of their hypotheses. Visscher was able to obtain variable results at will by varying the conditions during the precipitation of the blood proteins, or by changing the protein precipitant. Greater uniformity in results is apparent in the work of those who have examined ultrafiltrates or dialysates of blood or plasma, rather than filtrates. Foster (6), Lundsgaard and Holbøll, Anderson and Carruthers (2), Paul (12), Wright, Herr, and Paul (21), and Thomas found that the optical rotation of such preparations is usually less

than the reducing power. With the exception of Lundsgaard and Holbøll, these investigators did not observe a regular increase of the rotation of blood dialysates on standing, and do not admit the presence in normal blood of an unstable mutarotatory form of glucose such as was postulated by Winter and Smith.

The *in vivo* dialysate constitutes, we believe, a less objectionable medium for the comparison of the optical rotation and the reducing power of the normal blood sugar than those previously used. It was pointed out (14) that such dialysates present, when equilibrium is established, an accurate picture of the diffusible constituents of the circulating arterial plasma under normal physiologic conditions. No *in vitro* manipulation of the blood itself is concerned in their preparation. The content of glucose is approximately equal to that of the plasma; accordingly it is great enough for accurate polarimetric estimation. This determination is accomplished by direct observation of the clear, protein-free solution, without further concentration or treatment of any kind. The character and manner of preparation of the *in vivo* dialysate is such, therefore, that its study should afford the maximal opportunity for the detection of evidence suggestive of the presence in the blood of labile, optically active isomers of glucose.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Methods*</div>

Dogs under local anesthesia, or under general anesthesia with sodium isoamylethylbarbiturate, served as experimental animals. Dialysis was carried out in duplicate against modified Ringer's solutions containing initially no added glucose and 200 mg. of glucose in 100 cc. The resulting dialysates are designated Dialysate A and Dialysate B, respectively. The details of the technique and the chemical methods of analysis used have been described previously (14). The optical rotation of the dialysates was determined in the earlier experiments in a Schmidt and Haensch polarimeter with tubes 2 dm. long. In later experiments a precision instrument of the same make with tubes 4 dm. long was used. A mercury arc served as the source of light, which was passed through colored glass filters to isolate approximately the green line of wave-length 546 $m\mu$. Care was taken to secure uniform illumination of the polarimetric field, to verify the zero

point of the apparatus before and after each set of readings, and to insure the accuracy of the readings themselves. Since the filter system did not produce a completely monochromatic beam of light, a factor was employed to convert the observed angles of rotation to glucose concentrations. When 4 dm. tubes were used, the value of this factor was 0.412, corresponding to a specific rotation of 60.7° for glucose. Repeated determinations of the strength of 0.10 per cent solutions of glucose were accurate to within 1 to 3 per cent. The specific rotation of such dilute solutions of glucose was unaffected by the presence of salts, sodium bicarbonate, or acids. The temperature varied from 25–28°, but was not further controlled, for the specific rotation of glucose is not affected by such changes in temperature.

Results

The important point in the argument advanced by Winter and Smith, and by Lundsgaard and Holbøll, in favor of a new, labile form of glucose, was the spontaneous and regular increase in the optical rotation of their preparations to equality with the reducing power. It has been mentioned that the presence of this phenomenon has not been confirmed by many of the more recent investigators. The optical rotation of the *in vivo* dialysates obtained in the group of experiments designed to test this point is shown in Table I. The polarimetric observations were made with tubes 2 dm. in length, and were repeated on the different dialysates at the intervals indicated. The solutions were preserved with toluene. Some of the dialysates were kept in the refrigerator; others were allowed to stand at room temperature. Some variations in rotation from day to day were encountered, especially in those solutions which were somewhat low in glucose. The variations were not large, however, and were usually within the error of the polarimeter used. It may be concluded that the optical rotation of the *in vivo* dialysate is constant so long as bacterial effects are avoided. The average values obtained, reported in terms of glucose, were from 10 to 40 mg. lower than those for the reducing power, and seemed to bear no special relation to the level of the reducing power. The latter remained satisfactorily constant over long periods. Apparently substances likely to undergo spontaneous mutarotation were not present in these dialysates of the normal circulating blood.

It was found in accordance with observations of Anderson and Carruthers, that acidification of the *in vivo* dialysate produced a marked and immediate increase in the optical rotation. This phenomenon was investigated in a second series of experiments, the more sensitive polarimeter and observation tubes 4 dm. in length being used. The determinations of optical activity may

TABLE I

Changes in Reducing Power and Optical Rotation of in Vivo Dialysates on Standing (Calculated as Mg. of Glucose for Each 100 Cc.)

Experiment No.	Hours	Reducing power, Shaffer-Hartman method		Optical rotation	
		Dialysate A	Dialysate B	Dialysate A	Dialysate B
4	1	101	121	63	75
	24			68	81
	48			75	89
5	1	103	130	101	108
	24				
	40			89	110
	312	107	135	93	114
6	1			96	111
	24	130	130	100	105
	96			90	102
	168	127	127		
	192			95	91
7	1	210	212	164	181
	24			175	185
	72	210	210	171	175
9	1			86	96
	24	111	123		93
	96	103	132	83	90

accordingly be accepted as of considerably greater accuracy than those in the first series. The results of these comparisons are shown in Table II. As in Table I, it is seen that the initial rotation is always lower than the corresponding copper reduction value for glucose. After acidification the agreement between the two methods of analysis is complete. This is particularly satis-

factory when the polarimetrically determined values for glucose are compared with those for the fermentable reducing sugar. The differences, with the exception of Experiment 14 A, are scarcely greater than the expected analytic errors. Such precise correspondence between the fermentable reducing power and the optical rotation of the acidified dialysate, although possibly a mere coincidence, nevertheless strongly indicates the presence of but a single sugar, one having the properties of the ordinary α,β-glucose.

TABLE II

Total and Fermentable Reducing Power of in Vivo Dialysates and the Optical Rotation, before and after Acidification (Calculated as Mg. of Glucose for Each 100 Cc.)

Experiment No.	Reducing power, Shaffer–Hartman method		Optical rotation	
	Total	Fermentable	pH 8.0	pH 2.0
11 A	129	128	108	130
B	132	130	112	137
12 A	129	128	111	129
B	145	143	127	143
13 A	136	134	115	133
14 A	104	102		92
B	155	152	136	152
15 A	125		82	123
B	138		97	136

The immediate and marked change in rotation produced by acid can in no way be related to the presence of a labile glucose. Once such a substance is converted into ordinary glucose, it would be perfectly stable. The changes in the optical rotation of the *in vivo* dialysate produced by acid are, however, in part reversible. The initial pH of the dialysate is the same as that of the plasma, 7.3 to 7.5; this increases to about 8 with the loss of carbon dioxide to the air during the filling of the polariscope tubes. There is not much change in the optical rotation when mineral acid is added to produce a reaction of about pH 5. If more acid is added, the

increases in rotation become apparent immediately and are complete at a pH of 2.5 or less, the addition of excess acid having no further effect. Complete data on the changes in rotation with shifts in pH have not been obtained, for although the initial dialysate forms a clear solution at pH 8 it becomes unstable once the carbon dioxide has been driven off, and on attempting to readjust the reaction to pH 8 precipitation of calcium and magnesium salts occurs, with a consequent clouding that interferes with further polarimetric studies. In one experiment, however, the added acid was neutralized and the reversible nature of the change demonstrated. Similar reversibility of the change in rotation produced by acidification of ultrafiltrates of human plasma has been demonstrated by Wright, Herr, and Paul.

The reversibility of the changes produced in the optical rotation of the dialysates by the addition of acid indicates that the initial discrepancy between the optical rotation, and the reducing power of the dialysate, must be due to the presence of optically active substances other than α,β-glucose and not to a labile form of glucose. Assuming that the optical rotation of the acidified dialysate is due only to α,β-glucose, then the additional optically active constituents may be assumed to have the special property of possessing a measurable levorotation at pH 8 and a negligible or zero rotation at pH 2. A substance of this type is d-lactic acid, the salts of which are levorotatory while the free acid is feebly dextrorotatory. Abel, Rowntree, and Turner (1) isolated the zinc salt of "paralactic" acid from their *in vivo* dialysates, and more recently Wright, Herr, and Paul have shown that the zinc lactate isolated from glycolyzed blood is optically active and levorotatory. Table III shows that in the *in vivo* dialysate there is an apparent relationship between the amount of lactic acid present and the increase in the optical rotation produced by the addition of mineral acid. This increase was greatest in Experiment 15 in which the dialysate contained the most lactic acid.

The available data on the rotation of the salts of lactic acid, especially in very dilute solutions, are slight. It has been found that the levorotation of the salts increases with dilution, whereas the slight dextrorotation of the free acid diminishes. Yoshikawa (22) found $[\alpha]_D^{20} = -15.0$ to $-15.75°$ for solutions of lithium lactate containing the equivalent of from 2.37 to 0.476 gm. of lactic acid

for each 100 cc. Jungfleisch and Godchot (8) reported $[\alpha]_D^{15}$ = $-18°$ for a solution of zinc lactate equivalent to 0.389 gm. of lactic acid for each 100 cc. The specific rotation of the salts of lactic acid may thus vary with the type of salt, the concentration, and other undetermined factors. We have made a preliminary determination of the specific rotation of a somewhat more dilute solution as follows: pure zinc d-lactate equivalent to 100 mg. of lactic acid was dissolved in water, sodium hydroxide was added to pH 7.4 to precipitate the zinc, and the solution was made up to 100 cc. and filtered. The rotation of this solution in a tube 4 dm. in length at 26° and with the same lamp and light filters as in the studies on the *in vivo* dialysate, was $-0.071°$. After the

TABLE III

Levorotation Calculated from Lactic Acid Present, Compared with Observed Levorotation (Rotations Are Expressed in Terms of Mg. of Glucose in 100 Cc. of Solution)

Experiment No.	Lactic acid	Levorotation calculated	Levorotation observed*	
	mg. per cent			
11	12.0	4	25	22
12	7.0	2	16	18
13	10.0	3	16	
14	9.0	3	16	
15	40.0	12	39	41

* The difference between the rotations at pH 2.0 and pH 8.0.

addition of alkali to pH 8.6, the rotation was $-0.070°$, and after acidification to pH 2, $+0.003°$. The dextrorotation of the free acid under these conditions is negligible. The levorotation of the alkaline solution corresponds to a specific rotation of $-17.5°$, in terms of lactic acid and for the particular light used. According to the values given by Volk (19) for the rotatory dispersion of zinc ammonium l-lactate, this corresponds approximately to $[\alpha]_D$ = $-14°$, which is in fair agreement with Yoshikawa's determination on lithium lactate. This value, $[\alpha] = -17.5°$, being utilized, as the best available for the purpose, the effect of lactic acid on the rotation of the *in vivo* dialysate has been calculated, with the results shown in Table III. In Experiment 15 as much as 29 per cent of the initial levorotation may be ascribed to lactic

acid, but in the remaining experiments this proportion is much lower, due to the lower concentrations of lactic acid. It would appear that the effect of lactic acid may be considerably greater than is indicated by the data on its optical activity, or that the blood contains appreciable quantities of diffusible optically active substances besides lactic acid and glucose. Doubtless the amino acids, certain of which were isolated by Abel, Rowntree, and Turner, from *in vivo* dialysate of the blood will be found to be of importance in this connection.

Comment

The slight but definitely measurable effect of the small amounts of lactic acid present is in harmony with some of the preparations of Anderson and Carruthers in which glucose was removed by means of copper-lime precipitation. The resulting alkaline filtrates were levorotatory, but became dextrorotatory on acidification, as would be required if lactic acid was one of the chief optically active constituents. Study of the optical rotation after removal of the glucose by means of yeast fermentation, would appear to be of questionable value. Such a procedure has been used by Thomas, who found the resulting glucose-free dialysates to be optically inactive, as was also water similarly treated with yeast. He concluded, therefore, that the optically active constituents of his *in vitro* dialysates were completely fermentable, and therefore carbohydrate in nature, a conclusion which is not supported by the present experiments with *in vivo* dialysates. The hydrogen ion concentration of his solutions after fermentation was not stated, but possibly may have been sufficiently low to mask the optical activity of the lactic acid which must have been present.

The composition of ultrafiltrates of human blood may be somewhat more complex than *in vivo* dialysates of dog blood. According to the data of Paul, ultrafiltrates from the plasma of several normal persons disclosed not only much lower rotations in comparison with the reducing power, but also much greater variations from day to day than we have encountered with the *in vivo* preparations. Closer agreement between the rotation and reduction of ultrafiltrates of plasma of diabetic patients was obtained, but in a series of five cases of terminal uremia, relatively huge differences were found. In later experiments by Wright, Herr, and

Paul the optical rotation of ultrafiltrates from the plasma of twelve miscellaneous cases was studied before and after acidification. An increase in rotation was observed, just as in the present experiments, but in many cases this was so large that the final rotation in terms of glucose considerably exceeded the values determined by reduction.

Further investigation of these relationships is in progress, especially the optical activity of mixtures of glucose and lactic acid which are comparable in complexity with the dialysate of the blood. Until the effect of lactic acid can be more accurately assessed than is possible at present it would seem to be of doubtful value to catalogue or consider the effect of the various other optically active substances or systems, such as amino acids, that might be present.

SUMMARY

The optical rotation of the *in vivo* dialysate of normal circulating arterial blood in dogs is lower, in terms of glucose, than the copper reduction values for glucose. Significant changes in rotation on standing do not occur.

The addition of acid, to pH 2 or less, produces an immediate and complete increase in the optical rotation, so that the values for glucose, as determined by optical and chemical methods, are then in complete agreement. The ordinary α,β form of glucose is apparently the only fermentable reducing constituent present, and the presence of labile, mutarotatory forms of glucose in significant amounts can be excluded.

The presence of lactic acid accounts for some, although only for a relatively small part, of the increase in rotation caused by acid. It is suggested that lactic acid may play a greater part than is indicated by the available data on its optical properties, or that other substances or systems, which under the conditions of the experiments have optical properties similar to those of lactic acid, may be present.

BIBLIOGRAPHY

1. Abel, J. J., Rowntree, L. G., and Turner, B. B., *J. Pharmacol. and Exp. Therap.*, **5**, 611 (1914).
2. Anderson, A. B., and Carruthers, A., *Biochem. J.*, **20**, 556 (1926).
3. Barrenscheen, H. K., Kahler, H., and Hechl, H., *Biochem. Z.*, **167**, 77 (1926).

4. Denis, W., and Hume, H. V., *J. Biol. Chem.*, **60**, 603 (1924).

5. Eadie, G. S., *Brit. Med. J.*, **2**, 60 (1923).

6. Foster, G. L., *Proc. Soc. Exp. Biol. and Med.*, **21**, 210 (1923).

7. Greene, C. H., and Power, M. H., *Am. J. Physiol.*, **90**, 367 (1929).

8. Jungfleisch, E., and Godchot, M., *Compt. rend. Acad.*, **140**, 719 (1905).

9. Lundsgaard, C., and Holbøll, S. A., *J. Biol. Chem.*, **65**, 323, 343 (1925).

10. Maase, C., and Tachau, H., *Z. klin. Med.*, **81**, 1 (1914).

11. Nakahayashi, S., and Abelin, J., *Biochem. Z.*, **147**, 544 (1924).

12. Paul, J. R., *J. Clin. Inv.*, **3**, 631 (1927); **5**, 303 (1928).

13. Power, M. H., and Greene, C. H., *J. Biol. Chem.*, **74**, p. xix (1927).

14. Power, M. H., and Greene, C. H., *J. Biol. Chem.*, **94**, 281 (1931).

15. Stepp, W., *Ergebn. Physiol.*, **20**, 108 (1922).

16. Thannhauser, S. J., and Jenke, M., *Münch. med. Woch.*, **1**, 196 (1924).

17. Thomas, J., *Compt. rend. Soc. biol.*, **104**, 331 (1930).

18. Visscher, M. B., *Am. J. Physiol.*, **76**, 59 (1926).

19. Volk, H., *Ber. chem. Ges.*, **45**, 3744 (1912).

20. Winter, L. B., and Smith, W., *J. Physiol.*, **57**, 100 (1922).

21. Wright, S. L., Jr., Herr, E. F., and Paul, J. R., *J. Biol. Chem.*, **80**, 571 (1928).

22. Yoshikawa, J., *Z. physiol. Chem.*, **87**, 382 (1913).

IMPROVEMENTS IN THE PREPARATION OF
d-GALACTURONIC ACID*

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INTRODUCTION

Because of the inquiries we have received in the past 18 months from many investigators interested in obtaining galacturonic acid preparations, we have subjected the preparation of the pure acid to a critical study with the result that we now feel that its preparation is on a much firmer basis.

The method which we developed for the preparation of d-galacturonic acid (1), primarily from the pioneer researches of Ehrlich and his coworkers, had proved quite satisfactory in our laboratory as well as in the hands of others, but some investigators have experienced difficulties. The difficulties that these workers encountered in their attempts to prepare d-galacturonic acid from the lemon pectin preparation that we recommended are not surprising when it is borne in mind that the starting material was subject to variations in composition.

Some preparations contained 75 per cent of galacturonic acid anhydride; others in turn contained as high as 85 per cent of this constituent. We had observed that the barium salt of the galacturonic acid obtained from the pectin preparations with a low uronic acid anhydride content was often difficult to purify. This difficulty is without doubt to be attributed to the furan products formed by the prolonged action of sulfuric acid on the galacturonic acid liberated, and also to the formation of condensation products by the furfuraldehyde liberated from the arabinose fraction of the

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pectin molecule. Some aspects of this difficulty have been discussed in a paper by Link and Niemann dealing with the action of weak mineral acids on the uronic acids (2).

It is also significant that our experience and also that of others have shown that the crystallization of *d*-galacturonic acid, even when carried out under the most favorable conditions, is not a simple task. Thus, for instance, Ohle and Berend (3) reported this difficulty in one of their researches on the acetone derivatives of the sugars. They had succeeded in isolating galacturonic acid formed by the oxidation of diacetone galactose as the pure barium salt, but they did not succeed with the crystallization of the free acid when the barium salt was decomposed with dilute sulfuric acid. Ohle and Berend found it necessary to prove the identity of the galacturonic acid by converting the uncrystallizable syrup into the brucine salt of galacturonic acid which, fortunately, crystallizes readily.

One of the most significant changes which we have introduced is that we now use a pure polygalacturonide prepared by the California Fruit Growers Exchange, Research Department, as the starting material.[1] This polygalacturonide, which is distributed by the California laboratory under the name of citrus pectic acid, is prepared on a semicommercial scale by first extracting the pectin from the citrus pulp with acid. The acid extract is neutralized with calcium hydroxide and is then treated at 37° with dilute sodium hydroxide solution for about 10 minutes, whereupon the calcium pectate curd formed is converted into a state that permits easy handling. The alkali is removed with hydrochloric acid, the excess acid washed out, and the resulting pectic acid dried. The polygalacturonide (pectic acid) formed by this process is an admirably pure product. It is practically ash-free, and free from galactose and the pentosan araban. The galacturonic acid content of four different preparations, determined by the method developed in our laboratory (4), varied between 95.0 and 99.0 per cent.[2]

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[1] We have attempted to create the interest of several large chemical houses in the preparation of this acid. Unfortunately the preparation of the free acid on a large scale is unprofitable, due to the great difficulty encountered in the crystallization process.

[2] We wish to thank Mr. Bernard Burkhart for making the analyses.

The general course of the procedure used in the preparation of *d*-galacturonic acid from the polygalacturonide is in principle the same as that used in the preparative technique published recently (1). Since the success of the preparation is still dependent on manipulative minutiæ, it is advisable to publish the procedure in detail so that those not acquainted[3] with the rather sensitive chemical properties and idiosyncrasies of the uronic acids will experience no difficulties in repeating the work.

Preparation of Barium Salt of d-Galacturonic Acid

The hydrolysis is carried out most conveniently by introducing 80 gm. of the polygalacturonide into 4 liters of boiling 2.5 per cent sulfuric acid. The uronide is added in small portions so as to avoid the formation of a heavy gummy mass which has a tendency to adhere to the bottom of the flask and to char subsequently. It is advisable to add about a dozen glass beads to prevent bumping and to keep the gummy material from adhering to the bottom of the flask. The contents of the flask are then boiled under a reflux condenser for 15 hours. The temperature of the top layer of the solution in the flask should be between 100–101° throughout the course of the hydrolysis. To maintain this temperature an asbestos jacket is placed around the flask. We prefer an electric heater and advise against the use of a Bunsen burner as the heating unit, since the slightest overheating and charring make it difficult to obtain a pure barium salt.

After the termination of the hydrolysis period the solution is cooled and filtered to remove the unhydrolyzed fraction and the insoluble reversion products that are formed by the action of the sulfuric acid on free uronic acids (2). The filtration can be performed quite readily by using a mat of asbestos or paper pulp on a linen cloth in a Buchner funnel. The clear solution is usually colored lemon-yellow, unless it has been overheated. The sulfuric acid is removed in two steps. Approximately nine-tenths of the acid is removed by adding 290 gm. of $Ba(OH)_2 \cdot 8H_2O$ dissolved in 2500 cc. of water at 40°. The hydroxide should be added

[3] Many of the inquiries pertaining to the preparation of this acid have come from biochemists, bacteriologists, pharmacologists, and plant physiologists.

slowly while the solution is stirred vigorously with a mechanical stirrer to prevent local overheating and alkalinity. It is advisable to surround the vessel in which the neutralization is carried out with a water jacket so that the temperature does not rise above 40°.

After the barium hydroxide has been added, the solution is still acid to Congo red paper. 40 gm. of finely powdered barium carbonate (a slight excess) suspended in water are then added to complete the neutralization of the sulfuric acid and to convert the free galacturonic acid into the barium salt. The solution is next heated on a water bath at 70–80° for 20 minutes to insure the complete conversion of the galacturonic acid lactone into the barium salt. 10 to 15 gm. of a clarifying agent, like kieselguhr, filter-cell, or hy-flo,[4] and 5 gm. of activated blood charcoal[5] are now added and the heating continued for an additional 15 minutes. The stirring is continued throughout the neutralizing and clarifying operation.

The inorganic barium salts and the clarifying agents are removed by filtering the cooled solution through a Buchner funnel containing a mat of paper pulp or woolly asbestos[6] on a hardened filter paper. The contents of the Buchner funnel should be washed with 300 cc. of boiling hot water.

The resulting light coffee-brown colored solution is then concentrated to a volume of 200 to 300 cc. at 12 to 15 mm. pressure. The temperature of the water bath used to heat the flask should not exceed 50°. Under these conditions the temperature within the flask will be about 30–35°, which is essential to prevent the decomposition of the barium salt. The flocculent material and the inorganic barium salts that separate out while the solution is being concentrated are finally removed by filtering through an asbestos mat. The solution is then clarified again with 5 gm. of activated blood charcoal.[7] The light brown solution is now

[4] Filter-cell and the hy-flo are obtainable from the Johns-Manville Company, Chicago.

[5] The charcoal is activated by heating it for 6 hours at 125° followed by cooling in a desiccator.

[6] We recommend the grade C woolly asbestos manufactured by the Powhatan Mining Corporation, Baltimore.

[7] We have found Merck's acid-washed blood charcoal very efficient for this work.

poured slowly into 4 volumes of 95 per cent ethyl alcohol. The alcohol should be stirred vigorously. The freshly precipitated barium salt varies in color from light yellow to almost white. The purer the barium salt, the more slowly it separates from the alcoholic solution.

The separation is usually complete after 30 minutes standing. The barium galacturonate is filtered rapidly on a Buchner funnel to remove the diluted alcohol, whereupon it is partially dehydrated by washing first with 200 cc. of hot 95 per cent alcohol and then with the same quantity of hot 99 per cent alcohol. The alcohol is then pressed out by making use of a piece of rubber sheeting used in dental work. A piece of the rubber is fastened over the Buchner funnel with a rubber band and then pulled down firmly onto the barium salt by the suction from the pump.

The cake of partially dehydrated barium galacturonate is then transferred to a dry beaker containing 150 to 200 cc. of anhydrous ether and stirred until a fine flocculent state is reached again. The ether is then removed by filtration (again on a Buchner funnel). The drying of the barium salt is completed by spreading it out on a piece of filter paper in a vacuum desiccator containing calcium chloride.

The salt should emerge from the drying process in the form of a light yellow or colorless fluffy powder. The yield of the barium galacturonate from 80 gm. of the polygalacturonide is approximately 30 gm.—equivalent to 35 per cent of the theoretical quantity obtainable. The barium content should be between 26 and 27 per cent; theory demands 26.30 per cent barium. The optical rotation with water as solvent is $+ 25.0°$. The barium galacturonate does not have a sharp melting point even when pure, but begins to decompose at 180°.

The freshly precipitated barium galacturonate has a great affinity for water. Due to its hygroscopic properties, which are particularly evident immediately after it is filtered for the first time from the alcoholic solution, it is imperative to subject it to the dehydration process as quickly as possible. Discoloration usually takes place when the salt is not handled properly in the course of the drying operation. After it is finally obtained in a dry state, the tendency to become hygroscopic is much less, particularly when the salt is pure and free from the furan products formed in the course of the hydrolysis.

Conversion of Barium Galacturonate into d-Galacturonic Acid

22 gm. of the barium salt are dissolved in 600 cc. of water at 20°, after which 100 cc. of 95 per cent ethyl alcohol are added.[8] 360 cc. of 0.2 N sulfuric acid (slightly less than the theoretical quantity necessary) are next introduced in the course of 30 minutes from a burette. The solution is stirred vigorously throughout this time. After three-fourths of the acid has been added, an additional portion of alcohol (about 50 cc.) is added. Subsequent to the addition of the total quantity of acid 3 gm. of activated blood charcoal and 5 gm. of filter-cell are added, whereupon the solution is heated in a water bath at 50° for 15 minutes.

Then the inorganic barium salts, the charcoal, etc., are removed by filtering through a Buchner funnel containing an asbestos mat on a hardened filter paper. The solution should not be forced through the filtering medium too rapidly for the finely divided charcoal particles have a tendency to interfere with the crystallization process. The alcoholic solution is concentrated *in vacuo*. The temperature of the water bath should not exceed 40° at any time. When the contents of the flask are reduced to about 50 cc., the thin syrup is poured into 200 cc. of 95 per cent alcohol to precipitate the barium galacturonate that has not been decomposed. This salt is removed by filtration through a hardened filter paper and the solution concentrated again under reduced pressure to approximately 25 cc. If the clarification and filtration steps mentioned above have been conducted properly the solution will be a pale yellow color.

Crystallization of Galacturonic Acid

If the aforementioned conditions have been adhered to rigidly, the crystallization of the free acid can usually be accomplished most successfully by transferring the syrup to a round bottom crystallizing dish which is then placed in a vacuum desiccator containing fresh anhydrous calcium chloride. The desiccator is exhausted until the contents of the dish begin to boil under the reduced pressure; after standing under reduced pressure for 2 to 3 days, the crystallization will usually set in spontaneously.

If the crystallization does not begin spontaneously after the

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[8] The purpose of the alcohol is to reduce the tendency toward lactone formation. See Rehorst, K., *Ber. chem. Ges.*, **63**, 2279 (1930).

3rd day, it should be induced by either of the two following methods: Concentrate the syrup in the vacuum desiccator until it has the consistency of glycerol and then thin it out with 5 to 10 cc. of water and add enough 99 per cent alcohol to produce a slight permanent turbidity. Again place the dish in the desiccator and exhaust the air for a few minutes and allow to stand for 2 to 3 days.

Should this method fail, again concentrate the solution to a syrup in the vacuum desiccator and take it up in enough hot 70 per cent alcohol to effect complete solution. Then cool and add ether until a permanent turbidity is produced. If the solution is placed in a desiccator (not evacuated) containing small lumps of calcium oxide and P_2O_5 in flat vessels, the crystallization might set in in the course of 5 to 7 days—particularly if the desiccator is kept in a chamber below room temperature.

During the crystallization process the solution invariably acquires a reddish color. This is particularly the case when the crystallization has been successful from the outset. When the first attempt has failed, the solution rapidly turns to a dark brown color.

Once the crystallization has set in the solution should become practically solid. To purify the crystals the mother liquors are removed by filtering on a small Hirsch funnel and pressing them out thoroughly with the end of a spatula. They are then transferred to 15 to 20 cc. of 95 per cent alcohol in a small beaker, stirred for a few minutes, filtered, and washed free from whatever color still remains with small portions of alcohol. The washing is completed with absolute alcohol and finally with dry ether. The drying should be conducted at room temperature over P_2O_5 in a vacuum desiccator. If the preparation does not exhibit the correct melting point of 159–160°, it should be recrystallized from hot 90 per cent alcohol.

Galacturonic acid usually crystallizes from dilute alcohol or aqueous solutions in the α form as the monohydrate $C_6H_{10}O_7 \cdot H_2O$. This modification sinters at 110–115° and melts at 159–160°. Ehrlich and Schubert (5) have shown that the β form (the anhydrous variety) with a melting point of 160° can be obtained by boiling the monohydrate in absolute alcohol and then concentrating the alcoholic solution rapidly.

The average yield of the *d*-galacturonic acid monohydrate that we have obtained from over 50 different lots of the barium salt has been about 4 gm. per 22 gm. of salt. By salvaging the mother liquor[9] it has been possible to effect a slight increase in the yield, but usually it is more profitable to convert the uncrystallizable liquor into the barium salt again and repeat the conversion process.

SUMMARY

Precise directions for the preparation of crystalline *d*-galacturonic acid monohydrate by the hydrolysis of a polygalacturonide prepared from citrus pectin are given. The procedure outlined should make galacturonic acid available to those research workers who have heretofore experienced difficulty with its preparation.

BIBLIOGRAPHY

1. Link, K. P., and Dickson, A. D., *J. Biol. Chem.*, **86**, 491 (1930).
2. Link, K. P., and Niemann, C., *J. Am. Chem. Soc.*, **52**, 2474 (1930).
3. Ohle, H., and Berend, G., *Ber. chem. Ges.*, **58**, 2585 (1925).
4. Dickson, A. D., Otterson, H., and Link, K. P., *J. Am. Chem. Soc.*, **52**, 775 (1930).
5. Ehrlich, F., and Schubert, F., *Ber. chem. Ges.*, **62**, 1974 (1929).

. [9] The mother liquor contains the lactone of galacturonic acid which so far has not been obtained in a crystalline condition. It is a curious fact that whereas the lactones of glycuronic acid and mannuronic acid crystallize more readily than the free acids, the reverse is true in the case of galacturonic acid.

MICRO DETERMINATION OF AMMONIA IN THE MUSCLES OF THE FROG

By GUSTAV EMBDEN

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In a remark published in this *Journal*[1] Benedict and Nash criticize the determinations of ammonia, which were performed by my coworkers Riebeling and Selter and by myself[2] on the right and left gastrocnemius muscles of frogs.

Without going into the details of our method, partly misquoted by Benedict and Nash, I refer only to a paper recently published.[3] This paper, dealing with the same subject, entirely substantiates our former results.

I should be glad if the method criticized would now be reinvestigated by Benedict and Nash. In carefully following the procedure described by Embden, Carstensen, and Schumacher, I have no doubt they will duplicate entirely our statements.

[1] Benedict, S. R., and Nash, T. P., Jr., *J. Biol. Chem.*, **82**, 676 (1929).

[2] Embden, G., Riebeling, C., and Selter, G. E., *Z. physiol. Chem.*, **179**, 149 (1928).

[3] Embden, G., *Z. physiol. Chem.*, **196**, 23 (1931).

THE EFFECT OF DIET ON THE MANGANESE CONTENT OF MILK*

By A. R. KEMMERER and W. R. TODD

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Within the past year several workers have used milk as a manganese-low diet for studies on the rôle of this element in animal metabolism. Preliminary experiments by Waddell, Steenbock, and Hart (1) indicated that the addition of small amounts of manganese greatly improves the ovulation rhythm in female rats on a diet of milk, iron, and copper. Kemmerer, Elvehjem, and Hart (2) have shown that female mice reared on a similar diet fail to ovulate normally. When traces of manganese are added to this diet, the estrous cycles appear to be normal, and there is a distinct stimulation in the rate of growth. Mitchell and Miller (3) suggest that manganese appears to have some stimulating effect on growth and food intake when a milk diet is used.

Since cow's milk is being used so extensively in these experiments the question of the variation in the manganese content of milk becomes an important one. If the manganese content of milk is easily affected by the amount of this element in the ration, care must be exercised in choosing the milk used for this work.

The literature contains very few figures for the manganese content of milk. McHargue (4) found milk to contain 0.03 part of Mn per million. Skinner, Peterson, and Steenbock (5) reported the average amount of this element in the milk used in their experiments on the manganese metabolism of the rat to be 0.02 mg. of Mn per liter. In a later paper Peterson and Skinner (6) give the average for six samples of milk as 0.028 mg. per liter. Krauss (7) states that analyses of the fresh milk used in his experiments gave

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results ranging from 0.042 to 0.059 mg. per liter. Richards (8) gives the figure of 0.04 mg. per liter.

We are reporting in this paper the manganese content of milk from cows and goats when fed a standard ration and when given the same ration plus manganese.

<div align="center">EXPERIMENTAL</div>

The procedure used was very similar to that employed in the earlier work at this laboratory on the effect of the diet on the iron and copper content of milk (9, 10). Three high producing Holstein cows, receiving a standard ration of alfalfa hay, silage, and a

<div align="center">TABLE I</div>

Manganese Content of Milk from Cows before and after Supplementing the Ration with Manganese

Wks. on experiment	Mn per liter milk		
	Cow 1	Cow 2	Cow 3
	mg.	*mg.*	*mg.*
1	0.027	0.027	0.033
2	0.025	0.034	0.036
Average............	0.026	0.031	0.035
After addition of Mn			
3	0.026	0.034	0.031
4	0.026	0.026	0.030
5	0.041	0.032	0.031
6	0.028	0.029	0.028
Average............	0.031	0.031	0.030

grain mixture, were used for the studies on cow's milk. Samples of milk were taken weekly from each cow at individual milkings, placed in all-glass bottles, and taken directly to the laboratory for analysis. The manganese was determined according to the method outlined by Skinner and Peterson (11). When values for the milk during the basal period had been obtained, manganese additions were made to the ration of the three cows.

The individual constituents of the ration contained the following amounts of manganese: alfalfa hay 41.4 mg., fresh silage 9.9 mg., and the grain mixture 70 mg. of Mn per kilo. The calculated daily

intake for each animal was approximately 750 mg. of Mn. The manganese intake was increased to 5 times that of the basal ration by adding a 50 cc. solution containing 12.5 gm. of $MnSO_4 4H_2O$ to the grain mixture of each cow daily.

The results of the analysis of the milk from the three cows are given in Table I. The average manganese content of the milk when the cows were receiving the basal ration is 0.030 mg. per liter. The figures for the milk from the same cows when on a ration containing 5 times as much manganese are no higher than those for normal milk. These results show that the manganese content of milk cannot be influenced by increasing the manganese content of the

TABLE II

Manganese Content of Milk from Goats before and after Supplementing the Ration with Manganese

Wks. on experiment	Milk production		Mn per liter milk	
	Goat 1	Goat 2	Goat 1	Goat 2
	cc. per wk.	*cc. per wk.*	*mg.*	*mg.*
1	7190	6075	0.085	0.090
2	8110	3920	0.070	0.085
			360 mg. Mn added to ration	180 mg. Mn added to ration
3	7850	4390	0.077	0.084
4	7795	5255	0.096	0.076
5	6230	4250	0.093	0.083
6	5640	3940	0.124	0.119
7			0.112	0.105

diet within reasonable limits. The composition of the ration used for cows producing milk for manganese metabolism studies, therefore, is not an important factor. This does not mean that the amount in the milk used can be ignored because the amount may vary due to other factors such as the individual, the breed, and the period of lactation.

The goats were kept in individual pens and fed a ration of alfalfa hay and a grain mixture. They were milked twice each day and aliquots taken at each milking were composited for 1 week. The samples were preserved with manganese-free formaldehyde. The average daily manganese intake for these animals was 45 mg.

When values had been obtained for the basal period one of the goats was given 180 mg. of Mn and the other 405 mg. of Mn, in addition to that received in the ration. The results are recorded in Table II.

The average manganese content of goat milk is 0.082 mg. per liter. It is interesting to note that this figure is over 2 times the amount present in cow's milk. This fact is rather significant since no such differences were observed between cow's milk and goat milk in the case of iron and copper. The values for the milk after the goats were placed on the higher manganese levels are slightly higher than those for normal milk. The increase is especially noticeable during the 4th and 5th weeks on the supplemented ration. This difference may be due partly to the decrease in the milk production, because there was a definite decrease in the amount of milk produced during the latter part of the experiment. The milk production is included in Table II to show this decrease.

SUMMARY

1. Milk produced by cows on a normal ration contains approximately 0.03 mg. of manganese per liter.

2. The manganese content of cow's milk was not increased when sufficient manganous sulfate was added to the ration to increase the manganese intake 5-fold.

3. The manganese content of milk from goats on a normal ration is approximately 0.082 mg. per liter.

4. The addition of manganous sulfate in quantities sufficient to increase the manganese intake 5- to 10-fold increased the manganese content of goat milk very slightly.

We are indebted to Dr. C. A. Elvehjem for much help in the preparation of this paper.

BIBLIOGRAPHY

1. Waddell, J., Steenbock, H., and Hart, E. B., *J. Nutr.*, 4, 53 (1931).
2. Kemmerer, A. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 92, 623 (1931).
3. Mitchell, H. S., and Miller, L., *J. Biol. Chem.*, 92, 421 (1931).
4. McHargue, J. S., *J. Agric. Research*, 27, 417 (1924).
5. Skinner, J. T., Peterson, W. H., and Steenbock, H., *J. Biol. Chem.*, 90, 65 (1931).

6. Peterson, W. H., and Skinner, J. T., *J. Nutr.*, **4**, 419 (1931).
7. Krauss, W. E., *Ohio Agric. Exp. Station, Bull. 477* (1931).
8. Richards, M. B., *Biochem. J.*, **24**, 1572 (1930).
9. Elvehjem, C. A., Herrin, R. C., and Hart, E. B., *J. Biol. Chem.*, **71**, 255 (1926-27).
10. Elvehjem, C. A., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, **83**, 27 (1929).
11. Skinner, J. T., and Peterson, W. H., *J. Biol. Chem.*, **88**, 347 (1930).

THE ACTION OF MERCURIC SALTS ON CYSTINE*

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Vickery and Leavenworth (9) discovered that when silver sulfate acts on cystine in a weakly acid medium (pH 6.0) there is simultaneous oxidation and reduction of the cystine presumably according to the following equation.

$$3RSSR + 3H_2O = RSO_3H + 5RSH \tag{1}$$

The sulfonic acid (cysteic acid) was isolated in amounts varying from 0.3 to 1.7 per cent of the cystine taken instead of the theoretic 16.67 per cent. A small amount of ammonia nitrogen was found which indicated that some side reactions had occurred. Andrews and Wyman (1) offered evidence to show that much the same thing happens when cystine is precipitated with mercuric sulfate in sulfuric acid solution. Many workers had previously observed that the cystine was reduced after decomposition of the mercury precipitate, but attributed this to the reducing action of hydrogen sulfide. Andrews and Wyman (1) pointed out that such a reduction would result in the presence of free sulfur in the mercuric sulfide. They were unable to identify free sulfur in the precipitate from the action of hydrogen sulfide. They did find that the filtrate of the cysteine-mercuric sulfate precipitate had a rotation of +0.15° and the initial washings of +0.13° and assumed that this was due to the presence of cysteic acid. These two facts are evidence that mercuric sulfate in strongly acid solution acts on cystine in the same way as silver sulfate in weakly acid solu-

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tion. Preisler and Preisler (8) made a quantitative study of the action of silver sulfate on dithio compounds. They found that in the case of dithiodihydracrylic acid the reaction proceeds essentially according to Equation 1. Dithiodiglycolic acid, dithiodilactic acid, and cystine were found to form some of the corresponding thiol acids (RSH) but these reactions were not susceptible to quantitative analysis by the methods available. In a later paper Preisler (7) showed that the reaction of mercuric bromide, in the presence of mercurous bromide, with dithiodihydracrylic acid proceeds to the extent of at least 75 per cent according to Equation 1.

Inasmuch as mercuric sulfate has been widely used as a precipitant for cystine, a further study of this reaction was deemed worth while. It has been found that the reaction proceeds at least 75 per cent according to Equation 1. The nature of the products makes their quantitative separation difficult.

A series of experiments was performed in which mercuric sulfate in sulfuric acid solution was used to precipitate cystine. The precipitate, as noted by others, exhibited marked cohesive properties and was very difficult to filter and wash. In an attempt to obtain a granular precipitate, mercuric acetate alone, and mercuric acetate followed by mercuric sulfate, were used but both proved unsuccessful. Analysis of several cysteine-mercuric sulfate precipitates showed that large quantities of nitrogen other than cysteine nitrogen had been adsorbed by them. In this series only from 3 to 6 per cent of the total nitrogen was found in the filtrates. This small amount of nitrogenous substance was lost by adsorption on the barium sulfate when the sulfuric acid was removed with barium hydroxide. In addition to these difficulties the filtrates contained small quantities of cystine and cysteine which could not be completely removed. These obstacles were overcome by the following procedure.

The cystine was dissolved in a minimal amount of sulfuric acid; freshly prepared mercuric oxide was then added and the suspension stirred for half an hour. This was followed by the addition of an amount of sulfuric acid somewhat in excess of that required to convert the mercuric oxide to mercuric sulfate. A granular precipitate was obtained which could be easily filtered and washed. The filtrate contained 12 to 14.5 per cent of the total nitrogen.

After 72 hours of stirring no cystine or cysteine was found in the filtrate. With a shorter period of stirring the presence of these two compounds was noted while a longer period did not appreciably increase the nitrogen content of the filtrate. The mercury was removed from the filtrate with hydrogen sulfide and the sulfuric acid with basic lead carbonate. The lead salt of cysteic acid which was obtained by precipitation with alcohol varied in composition within such wide limits that the attempt to isolate the cysteic acid from these filtrates as the lead salt was abandoned. Instead the lead was removed with hydrogen sulfide and the filtrate was taken almost to dryness in a vacuum. Absolute alcohol was then added and the product was taken to dryness in a vacuum. This

TABLE I

Formation of Cysteic Acid from Cystine

Experiment No.	Cystine	Total N	N in filtrate		N in precipitate		Crude cysteic acid	Cystine recovered	
	gm.	gm.	gm.	per cent of total	gm.	per cent of total	gm.	gm.	per cent of total
1	10.000	1.168	0.158	13.6	1.044	89.6		7.80	78.0
2	10.000	1.168	0.166	14.2				7.15	71.5
3	10.000	1.168	0.148	12.7	1.010	86.5	0.95	7.48	74.8
4	20.000	2.336	0.283	12.1	1.953	83.8	1.57	15.05	75.3
5	20.000	2.336	0.337	14.4	1.885	80.8	3.44	15.18	75.9
6	20.000	2.336	0.310	13.3	2.050	88.0	3.02	15.15	75.7
7	20.000	2.336	0.315	13.5	1.965	84.2	3.28	15.65	78.2

treatment was repeated until the cysteic acid separated as a white precipitate. As a check on this procedure, a known amount of cysteic acid in sulfuric acid solution was treated with basic lead carbonate and the filtrate was decomposed with hydrogen sulfide. After the lead sulfide had been filtered off, the filtrate was concentrated in a vacuum and then treated with alcohol, as described. The recovery of pure acid was 92.5 per cent of the theoretic.

The results of a series of experiments in which cystine was treated with mercuric oxide and sulfuric acid are given in Table I. The nitrogen content of the filtrates is from 12 to 14.5 per cent of the total nitrogen. This is from 72.1 to 87.0 per cent of the theoretic according to Equation 1. It is to be noted that in all these filtrates not more than a trace of ammonia nitrogen was found.

The total ammonia was not more than 1 to 2 mg.   Vickery and Leavenworth (9) found ammonia equivalent to 1 to 2 per cent of the original cystine nitrogen in their cysteine-silver sulfate filtrates.

Decomposition of the cysteine-mercuric sulfate precipitate with subsequent determination of the cysteine as cystine, resulted in recovery of from 71 to 78 per cent of the original cystine.   Analysis showed that the crude cysteic acid isolated was 90 to 95 per cent pure.   The nitrogen content was close to the theoretic whereas the sulfur was low.   This indicated the presence of a nitrogenous impurity.   The cysteic acid was purified by reprecipitation from aqueous solution with alcohol or by fractional crystallization.   One sample purified by the latter method gave a product which was 98 per cent pure.

TABLE II

Analysis of Cysteic Acid

	Nitrogen	Sulfur	N NaOH used in titration	
			Sulfonic group (84.6 mg.)	Carboxyl group (84.6 mg.)
			cc.	*cc.*
Calculated for $C_3H_7O_5NS$......	8.28	18.95	0.500	0.500
Sample 1.....................	8.53	17.56	0.444	0.443
" 2.....................	8.25	17.74	0.467	0.474
" 3.....................	8.27	18.66	0.491	0.489

Table II contains the analyses of the different samples of cysteic acid isolated.   Samples 1 to 3 are those isolated from Experiments 4 to 6, respectively.   Samples 1 and 2 were purified by reprecipitation from aqueous solution with alcohol, and Sample 3 by fractional crystallization.

It will be noted that although the total nitrogen in the mercury precipitate and filtrate approaches very closely the theoretic, the combined nitrogen value of the cystine and cysteic acid does not. Some of the cysteic acid formed no doubt remains with the cysteine-mercuric sulfate precipitate and is lost in this manner.   The recovery of cystine is very consistent and averages 75 per cent of the original cystine instead of the theoretic 83.33 per cent.   This discrepancy is mostly due to the adsorption of cysteine on the

mercuric sulfide precipitate. A known sample of cysteine was precipitated with mercuric sulfate in sulfuric acid solution. The recovery of cysteine after decomposition of the precipitate was 93.5 per cent.

The mechanism suggested by Preisler and Preisler (8) implies an oxidation of sulfate ion by the dithio compound. Compounds of the type RS—SR are such very weak oxidizing agents that under the conditions of the experiment they would certainly not be able to oxidize a sulfate to a persulfate.

A possible mechanism is the following. It is assumed that RS—SR dissociates slightly into RS$^-$ and RS$^+$. On addition of the salt the negative ion is removed from the field of action by the metal ion (Ag$^+$, Hg^{++}, and so forth) as the insoluble metal-cysteine compound. The positive ion may then add a hydroxyl group to form a derivative of hydrosulfoxylic acid, which is a strong reducing agent, or it may act directly on more of the disulfide. In either case the sulfur acts as a powerful reducing agent with the result that two disulfide groups are reduced to sulfhydryl while the reducing agent is oxidized to sulfonic acid.

Experimental Procedure

20.000 gm. of cystine, prepared by the method of Gortner and Hoffman (4), were dissolved in 55 to 60 cc. of 5 N sulfuric acid. Chloride-free mercuric oxide, prepared from 100 gm. of mercuric chloride and 150 cc. of 5 N sodium hydroxide, was added, the suspension diluted with water to 2000 to 2500 cc., and then stirred for ½ hour. At the end of this time 170 cc. of 5 N sulfuric acid were added and the mixture stirred for 72 hours by means of a mechanical glass stirrer. The precipitate was filtered off with the aid of suction and washed well with water. The filtrate and precipitate were treated as follows:

1. *Cysteine-Mercuric Sulfate Precipitate*—The precipitate was suspended in 1500 cc. of water and decomposed with hydrogen sulfide. The mercuric sulfide was removed by filtration and the filtrate concentrated in a vacuum to a small volume. An aliquot was made barely alkaline with barium hydroxide and then aerated until the nitroprusside reaction was negative. Cystine was then determined by a modification of the Folin-Looney procedure (3).

2. *Mercuric Sulfate Filtrate: Isolation of Cysteic Acid*—The fil-

trate and washings were concentrated in a vacuum to a volume of 500 cc. and a portion was titrated for its acidity with N sodium hydroxide. A small excess of basic lead carbonate (freshly prepared and washed until free of nitrates) in addition to that required to neutralize the sulfuric acid was added and the suspension refluxed for 1 hour. After cooling, the mixture was filtered and the lead precipitated with hydrogen sulfide. The filtrate after removal of the lead sulfide was taken almost to dryness in a vacuum. Then 25 to 50 cc. of absolute alcohol were added and the product taken to dryness. The addition of alcohol was repeated until the precipitate became dry and fell to the bottom of the flask. The product was dissolved in the least possible amount of water and cooled in an ice-salt mixture. The cysteic acid that crystallized out at this point, on analysis, was found to be 98 per cent pure.

The nitrogen was estimated by the Koch-McMeekin micro-Kjeldahl method (6) and the sulfur by the Benedict method (2). The sulfonic and carboxyl groups were estimated by the procedure of Harris (5), brom-thymol blue being used as indicator.

SUMMARY

It has been shown that when mercuric sulfate in sulfuric acid solution acts on cystine the reaction proceeds to the extent of at least 75 per cent according to the equation

$$3RS\text{—}SR + 3H_2O = RSO_3H + 5RSH$$
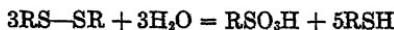
The sulfonic acid (cysteic acid) was isolated in amounts up to 68 per cent of the theoretic amount.

A possible mechanism for the reaction is offered.

BIBLIOGRAPHY

1. Andrews, J. C., and Wyman, P. D., J. Biol. Chem., 87, 427 (1930).
2. Benedict, S. R., J. Biol. Chem., 6, 363 (1909).
3. Folin, O., and Looney, J. M., J. Biol. Chem., 51, 421 (1922).
4. Gortner, R. A., and Hoffman, W. F., in Marvel, C. S., Organic syntheses, New York, 5, 39 (1925).
5. Harris, L. J., J. Biol. Chem., 84, 296 (1929).
6. Koch, F. C., and McMeekin, T. L., J. Am. Chem. Soc., 46, 2066 (1924).
7. Preisler, P. W., J. Biol. Chem., 92, p. xxxvi (1931).
8. Preisler, P. W., and Preisler, D. B., J. Biol. Chem., 89, 631 (1930).
9. Vickery, H. B., and Leavenworth, C. S., J. Biol. Chem., 86, 129 (1930).

LUCIUS LINCOLN VAN SLYKE

We regret to record the death of Lucius Lincoln Van Slyke who served for many years on the Editorial Committee of The Journal of Biological Chemistry.

Dr. Van Slyke was born in Centerville, New York, on January 6, 1859, and died in Geneva, New York, on September 30, 1931. He studied at the University of Michigan and received the degree of A.B. in 1879, A.M. in 1881, and Ph.D. in 1882. In his early scientific career Dr. Van Slyke served as Instructor in Analytical Chemistry at the University of Michigan, as Professor of Chemistry in Oahu College, Honolulu, and as Government Chemist in the Hawaiian Islands. After two further years spent at the University of Michigan and the Johns Hopkins University, Dr. Van Slyke came to the New York State Agricultural Experiment Station at Geneva in 1890 as Chief Chemist. He continued in this post for thirty-eight years until he retired from active duty in 1928.

His first work at the Experiment Station was concerned with the introduction and development in the State of New York of chemical inspection of fertilizers and feedingstuffs, but his chief interest was in the field of research. For many years he devoted himself to investigations in dairy chemistry, particularly of milk products, cheese, and the chemistry of casein. On these subjects he contributed many important papers to scientific journals as well as to numerous Experiment Station bulletins. In addition, he was the author of "Modern methods of testing milk and milk products," "The science and practice of cheese making," "The chemistry of milk and milk products," and "Fertilizers and crops."

In the death of Dr. Van Slyke the country has lost one of the leading figures in agricultural and dairy chemistry. During his long and distinguished service as Chief Chemist at the Experiment

Station at Geneva he contributed greatly to the advancement of scientific agriculture and to dairy science.

As a man, Dr. Van Slyke was held in the highest esteem by his friends and colleagues. He had an alert mind, was widely read, and had a delightfully keen sense of humor. He interested himself not only in the development of his own special field in science, but took an active part in the life of the community around him. He was an expert in floriculture and in gardening; he loved music; and he supported his church as ardently as he did all movements toward civic improvements. He was a gentleman of the old school, honest and upright, and an outstanding personality in his community.

THE CORRELATION BETWEEN RATE OF OXIDATION AND POTENTIAL IN IRON SYSTEMS

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It is well known that in general the rate of a chemical reaction has no relation to the free energy change accompanying this reaction. The change of free energy depends only on the state of the reactants before and after the reaction concerned. The rate, however, depends on the intermediate states also and can be varied by catalysts. If, however, we take any given reaction and break it up into sufficiently elementary processes we must find one process for which there are no intermediate steps and for this process the rate might be in some way or other intimately related to the free energy change. As far as biological oxidations are concerned we know that in many cases one intermediate step seems to be the formation of an iron, or at least some heavy metal, complex. This is followed by a change of valence on the part of the iron. This change of valence is a very elementary process and it is this process with which the present paper is concerned. We shall endeavor to show that the rate at which this process occurs in a few simple systems runs parallel with the normal potential of the system in which the process occurs.

During the course of previous work (1, 2) it became apparent that the order of the normal potentials of various reversibly oxidizable and reducible iron complex systems closely paralleled the order of the rates at which the ferro members of the systems were oxidized by molecular oxygen. By the normal potential of a system we mean the potential of a solution containing equal parts of the ferro and ferri forms of the complex. This normal potential depends on pH so we wish to restrict our comparison between the

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potential and the rate of autoxidation to iron systems at constant pH. It is necessary, of course, that the pH chosen be compatible with the existence of the complex.

It is not asserted that in general potential and rate run parallel. Our assertion is that if we examine the *normal* potentials of a series of iron complex compounds at any constant pH, the order of the potentials is the same as the order of the rates of autoxidation of the ferro form of these complexes. The more negative the potential, the more rapid is the oxidation by molecular oxygen.

In a previous paper (1) it was shown that the rate of oxidation of ferrous compounds by molecular oxygen was greatly dependent upon the electrical condition of the ferrous iron. Ferrous ion was oxidized much more slowly than the electrically neutral ferrous iron atom contained in various compounds. In a subsequent paper (2), the normal potentials of various iron complex systems were investigated. Before demonstrating the validity of the above rule for these data we shall consider certain iron complexes not investigated in the earlier papers, for these cases will be particularly demonstrative.

First we may consider the case of some cyanide complexes of iron. As previously pointed out (1), although in ferrocyanide the iron atom is tightly held in an unionized condition it is still difficult for it to be oxidized. This was considered to be due to the fact that the iron atom is surrounded by four negative charges and in order for an electron to escape from the kernel of the iron it must move out against this negative atmosphere. This is a different consideration than the charged condition of the iron atom itself and one cannot, offhand, predict how important it will be in comparison with positive charges on the iron. However, the only thing that need concern us here is whether or not the autoxidizability and the normal potential run parallel. We have seen that ferrocyanide is not readily autoxidizable; then it should be a comparatively poor reducing agent and its normal potential should be relatively positive. As Fig. 1 shows, the potential is quite positive. As the normal potential of a system containing such polyvalent ions is very sensitive to changes in the ionic strength we have to make the comparison of various compounds of this kind at a constant ionic strength. We shall compare them under the conditions established by dissolving a very small amount

of the complex in a 1.0 M KCl solution.   Under these conditions
the potential was found to be +0.480 volt when expressed against
the normal hydrogen electrode.

It is possible to substitute one of these cyanide groups of ferro-
cyanide for any one of several other groups and Davidson (3)
has measured the potentials of some of the derivatives.   We would
expect from our considerations that the substitution of an NO_2^-
group for a CN^- group would not change the potential very mark-
edly and to the extent that it did change it we would expect the
value to become more positive.   The value reported by Davidson,
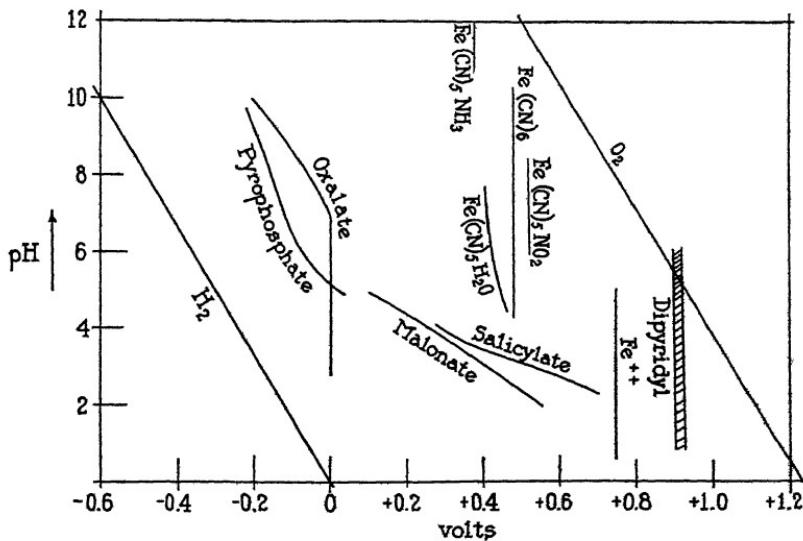
FIG. 1.   The normal potentials of various iron systems

0.516 volt (3), is in fact somewhat higher.   We would also expect
that the replacement of a CN^- group by an NH_3 group would
appreciably lower the potential, for this reduces the negative
charge of the molecule by 1 unit.   The value found by Davidson,
when measured in an excess of ammonia, was 0.374 volt (3).
Likewise we would predict that if a CN^- group is replaced by H_2O
the resulting potential will be considerably lower and the complex
in its ferro form will be more inclined to autoxidation.   The latter
is true.   The potential, however, according to Davidson is not
more negative than that of ferrocyanide, but even more positive.

He reports the potential of this aquo compound to be 0.491 volt (3). This value is higher than that of the ferrocyanide. Of the various compounds to be considered in this paper this penta-cyanoaquoferroate is the only one that is out of its predicted place. We may then justifiably harbor a suspicion concerning the accuracy of this value. This suspicion is further increased by the fact that Davidson reports difficulty in measuring this potential. He attributes this difficulty to the formation of an intermediate compound containing 3 molecules of the oxidized and 2 of the reduced form.

On the basis of the above suspicion we have repeated the potential measurements on this compound and for the reasons fully set forth in the experimental part we believe that Davidson's value is in error. As pointed out later, the assignment of an E_0 value to such an irregular system involves some assumptions. If these assumptions be granted then our E_0 for the system is 0.418 volt at pH 6.6. This value puts this system in its expected place in our arrangement (Fig. 1). Even if this value is not correct our results clearly show that Davidson's value is much too high and that this system is, at least, not a contradiction of our rule. Without insisting too much on the fact that our corrected potential value shifts this complex to the expected place in the order of autoxidizability, one can say that this complex is not a simple complex with only one iron nucleus and therefore not comparable with the others and not suitable for the application of our rule.

A corollary of the theory proposed is that all complex ferro cations, in which the iron has retained its positive charge, should be difficult to oxidize, and that a mixture of these with the corresponding ferri complexes should have a very positive potential. One beautiful example demonstrates the validity of this statement. The ferro complex of α, α'-dipyridyl, investigated by Blau (4), when written in modern fashion, according to Werner's scheme, has the constitution shown in Formula I.
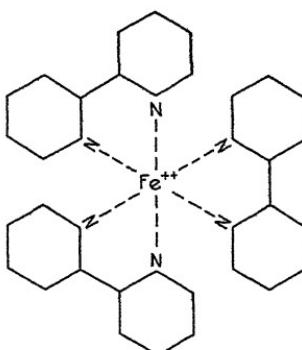
Formula I

This complex is entirely stable toward oxygen and so difficultly oxidizable that not even bromine, but only chlorine or permanganate, can oxidize it to the ferric state. Exposure to the air even in alkaline solution for any length of time does not oxidize this red ferro complex into the blue ferric.

We attempted to measure the potential of the dipyridyl complex by titrating ferrous dipyridyl with an aqueous solution of chlorine. The potential range was very positive indeed, being much more positive than 1.0 volt, even much more positive than that of the system ferric chloride + ferrous chloride, but no steady potential could be obtained. These unsteady potentials may be due in part to the fact that all noble metal electrodes no longer work as indifferent reversible electrodes when the potential range approaches that of the oxygen electrode and in part to the fact that the ferri complex is not a very stable compound but liable to intramolecular rearrangement in such a way that the organic part of the complex is oxidized and the iron reduced to the ferro state, as has been observed by Baudisch (5). Another contributing cause may be that the chlorine used as oxidant will in part directly attack the organic part of the molecule in addition to oxidizing the iron.

Now we may draw a graph (Fig. 1) comprising all potentials of the various systems, including those just discussed. We do not have measurements for all the systems at any one pH, but we can compare the range of the potentials, say at pH 5.0. The order of the potentials is: dipyridyl, chloride or sulfate, $(CN)_5NO_2$,

$(CN)_6$, $(CN)_5H_2O$, $(CN)_5NH_3$, salicylate, malonate, oxalate, pyrophosphate. This is precisely the order of autoxidizability. The ferro dipyridyl complex is not autoxidizable at all, the free ferrous ions of an acidulated solution of ferrous sulfate, as well as the ferrocyanide complex, are very stable, but the next, pentacyanoaquoferroate and pentacyanoaminoferroate, are definitely autoxidizable, though at a relatively slow rate. Ferrous salicylate and malonate are readily autoxidizable. A solution of ferrous salt dissolved in an excess of salicylate at pH 5.0 and exposed to air gradually develops the violet color of the ferri complex. The rate of this oxidation is of a medium, measurable order of magnitude. Ferrous oxalate and pyrophosphate absorb oxygen so rapidly that the rate can scarcely be measured in a micro respiration apparatus. Thus, although we cannot offer precise quantitative data for the different rates of autoxidation, the order of magnitude differs so largely that the validity of our statement is obvious. At any other pH some of the compounds can be compared with each other in a similar way and with the same result. As we compare various iron complexes at any other constant pH, the number of such complexes compatible with this pH may be smaller but those available for a comparison will always be in the correct order.

The physiological significance of the rule stated above may be expressed as follows. The various porphyrin iron complexes fulfil at least two different functions. Some of them serve as oxygen carriers, others as catalysts for oxidation of other substances. The best known oxygen carrier, hemoglobin, is a ferro compound. To fulfil its function as oxygen carrier, the iron of hemoglobin must not be oxidizable by molecular oxygen. The oxygen compound of its ferro state must be stable in this ferro state. On the other hand the catalytically acting heme pigments can fulfil their function only if their iron nucleus is autoxidizable in a reversible way. We must then distinguish autoxidizable and non-autoxidizable heme pigments. Thus hemoglobin is not autoxidizable, but denatured hemoglobin is. Such considerations suggest the question, upon what measurable properties does the autoxidizability of an iron complex depend? It seemed desirable to investigate this problem for simpler iron complexes with the hope that whatever results were obtained could be applied to the heme pigments later. Potentiometric measurements on the heme pigments have

been performed by Conant and coworkers (6) but the data presented by these authors do not seem to be sufficiently definite to justify the application of the principle stated above, so we must restrict ourselves to merely mentioning the physiological point of view which underlies the rule stated in this paper.

<div align="center">EXPERIMENTAL</div>

The sodium pentacyanoaquoferroate $[Fe(CN)_5H_2O]Na_3$ was prepared by treating sodium nitroprusside with hydroxylamine
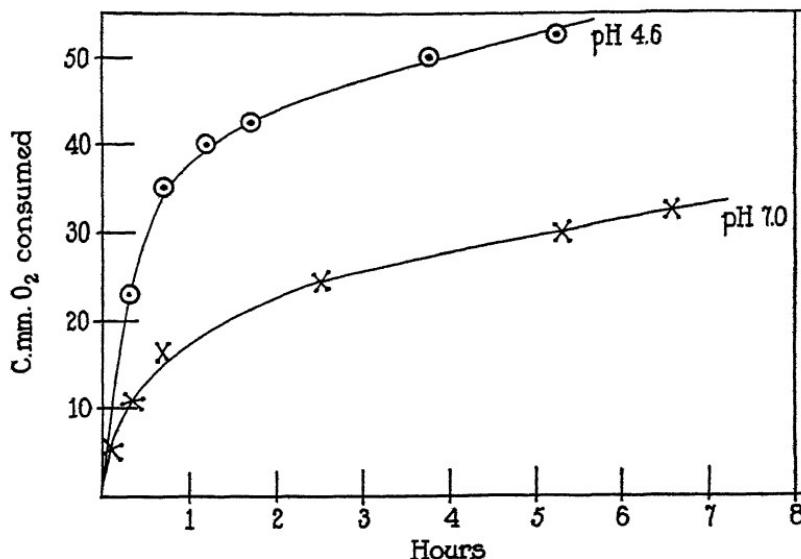
FIG. 2. Oxygen consumption of sodium pentacyanoaquoferroate solution. The experiments were carried out in a Warburg respiration apparatus at 22°. Each flask contained 1.6 cc. of 0.02 M solution. If the solution is made more acid than about pH 4.0 the rate of oxidation again decreases. This decrease of autoxidizability is accompanied by a color change of this ferro solution from the usual yellow color to a blue-green color, similar to the color of the ferri compound.

hydrochloride according to the method of Hofmann (7). The corresponding ferri compound was prepared by oxidizing the ferro compound with bromine water at low temperature, again in the manner described by Hofmann (7). These procedures led to a yellow powder for the ferro preparation and a very dark blue

powder for the ferri compound.   The aqueous solutions have about
the same colors as the powders.

In agreement with Davidson (3) it was found that when a
solution of the ferri compound was mixed with an excess of the
ferro compound the color of the ferri solution immediately faded
out.   This is not due to an optical mixing of the colors, as can
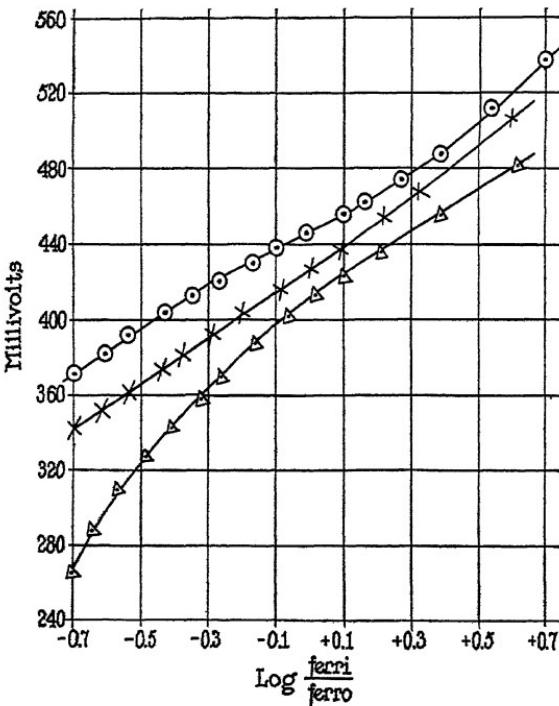
Fig. 3.   The effect of pH on the potential of the system sodium penta-
cyanoaquoferroate-sodium pentacyanoaquoferriate, established by mixing
the two compounds.   ⊙ pH 4.6 (0.2 M acetate buffer), × pH 6.0 (0.2 M
acetate buffer), △ pH 8.0 (M/15 phosphate buffer).

easily be determined, but to some intermediate compound for-
mation.

In working with systems such as this there are several factors
that must be closely controlled.   The first, which has already
been discussed, is change in ionic strength.   Another danger arises
from the fact that the ferro compound is oxidized by the oxygen

of the air.   This autoxidizability is shown in Fig. 2.   Such autoxidizability itself may be taken as an indication of the fact that the potential of the system is more negative than that of the ferrocyanide system.   If this autoxidizability is not guarded against, its effect on the titration curve obtained by mixing the two forms is to flatten it at both ends and to place the entire curve in a too
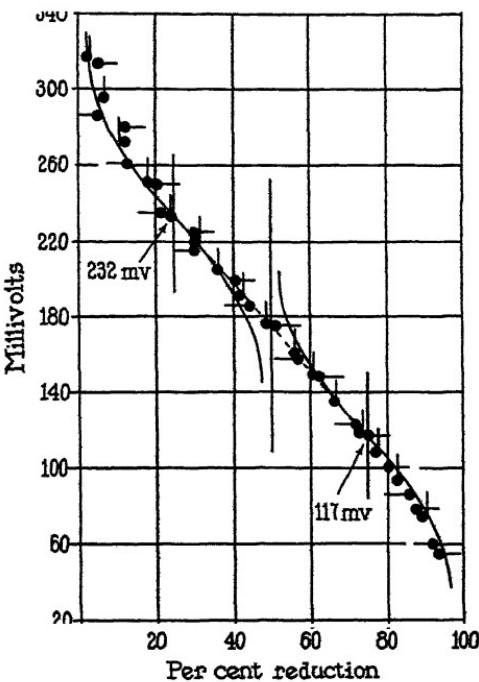
FIG. 4.   The titration of sodium pentacyanoaquoferroate with reduced rosinduline.   ⬇ 0.16 mM in volume of 25.0 cc., ●— 0.08 mM in volume of 25.0 cc., —● 0.008 mM in volume of 25.0 cc.   The curves drawn are those calculated for two steps of oxidation, each involving 1 electron.   To refer these potentials to the normal H_2 electrode add 242 millivolts.

positive range, since the ratio of ferri to ferro is always greater than that calculated.   The third factor that must be controlled is the pH.   This was not done in Davidson's work.   In the case of ferrocyanide it is not important to work at constant pH, for the potential of this system, at pH more than 5.0, is independent of pH.   However, this is not true for the aquo compound under consideration as Fig. 3 shows.   The solutions of the ferro and ferri

compounds have quite different acidities.   Measurements with a glass electrode showed that a 0.01 M solution of the ferro compound had a pH of 8.5.   A similar solution of the ferri compound had a pH of 6.0.   If one wishes to maintain a constant pH during the mixing of such solutions the necessity for buffering is evident.   A small part of the difference shown by the curves in Fig. 3 may be due to changes in ionic strength, but since all solutions were 1.0 M with potassium chloride this change would be small.

In order to meet the above requirements we chose the following conditions.   The ferri compound to be used was weighed out and dissolved in 1.0 cc. of water.   To this were added 3.0 cc. of a M/15 phosphate mixture and 22.0 cc. of 1.0 M KCl.   The pH of such solutions was 6.6.   This was titrated with a solution of reduced rosinduline of appropriate concentration.   The results obtained are shown in Fig. 4.

The most striking thing about the curves is that they are much too steep as compared with those of other complex iron systems. The change from the ferri to the ferro form should be a 1 electron change and this should correspond to a potential change of approximately 30 millivolts for a 3-fold change in the ratio of the ferri to the ferro form.   The actual potential change is about twice this.   The only apparent explanation for this is that both the ferri and the ferro compounds exist in solution in a polymerized bimolecular form and that an intermediate compound containing both ferri and ferro molecules also exists.   From the fact that the curves are symmetrical about the mid-point we may assume that the intermediate compound present at this point contains the ferri and ferro forms in equal proportions.   If this be true then the potential at this point is the E_0 of an imaginary system containing only the ferri and the ferro forms of the complex.   As may be seen, this is 0.418 volt at pH 6.6.   If we consider the E_0 potential for the first step consisting of the oxidation of the bimolecular ferro compound to the mixed ferro-ferri compound, this is even much more negative.

The three experiments in Fig. 4 represent three different concentrations.   Apparently, from the mid-point onward the three curves are accurately the same, but from the beginning to the mid-point there is considerable deviation.   This finds a ready explanation if we assume that the polymerized ferri form is in equilibrium with a

monomolecular ferri form.   Any change in concentration or other conditions would affect this equilibrium and hence the potential.

If we employ the same conditions we can duplicate the curves shown, either by reducing the ferri compound with reduced indigo tetrasulfonate instead of rosinduline or by mixing the ferri and ferro compounds.   It is more difficult to obtain as good results by oxidizing the ferro compound for at the temperature employed (30°) no good end-point is obtained, probably due to irreversible oxidation of the cyanide by the strong oxidant which it is necessary to employ (Cl_2 or Br_2).   In Fig. 4 the results for this complex are plotted for three experiments with varied initial concentration of the ferri compound which is titrated with reduced rosinduline as a reductant.   The drawn out curves are calculated on the assumption that there are two steps of reduction each involving the addition of 1 electron without any change in the molecular size. The agreement of the calculated and the observed values is certainly not as good as usual in stable reversible systems.   The slight though obvious dependence of the upper end of the curve on the initial concentration was consistent in repeated experiments.   On considering the instability of this complex one might be inclined to attribute these deviations to impurities due to secondary decomposition products.   If this be true the only possible interpretation is that this complex is bimolecular and exists in the ferro-ferro, ferro-ferri, and the ferri-ferri states.   1 molecule of H_2O may be considered to function as a bridge holding together two $Fe(CN)_6$ radicals.

It is scarcely thinkable that the slight deviations from the theoretical curve might require a complete abrogation of this interpretation.   It cannot be decided however whether the deviations are due to the mentioned impurities or to the fact that the association of each 2 molecules to a double molecule is not entirely complete but in equilibrium with the monomolecular state. This would involve a dependence of the potential on the absolute concentration of the substance, as has been pointed out in previous papers.

SUMMARY

It is shown that for a series of iron compounds the autoxidizability of the ferro compound at a given pH closely parallels the

normal oxidation reduction potential of the system ferro compound-ferri compound, at the same pH. The more negative the potential, the greater is the autoxidizability.

It is shown that the normal potentials of various iron systems reported in the literature are, with one exception, in good agreement with the above statement and with the potentials expected from a knowledge of the structure of the compounds.

The potential of the system sodium pentacyanoaquoferroate-sodium pentacyanoaquoferriate, the exception noted above, has been reinvestigated. We are unable to confirm Davidson's value of 0.491 volt, expressed against the hydrogen electrode. Our data indicate that this is a very irregular system and the interpretation of the results involves some assumptions. These assumptions being granted, the exceptional position of this complex vanishes.

BIBLIOGRAPHY

1. Smythe, C. V., *J. Biol. Chem.*, **90**, 251 (1931).
2. Michaelis, L., and Friedheim, E., *J. Biol. Chem.*, **91**, 343 (1931).
3. Davidson, D., *J. Am. Chem. Soc.*, **50**, 2622 (1928).
4. Blau, F., *Monatsh. Chem.*, **19**, 647 (1898).
5. Baudisch, O., *Biochem. Z.*, **92**, 189 (1918).
6. Conant, J. B., *J. Biol. Chem.*, **57**, 401 (1923). Conant, J. B., Alles, G. A., and Tongberg, C. O., *J. Biol. Chem.*, **79**, 89 (1928). Conant, J. B., and Tongberg, C. O., *J. Biol. Chem.*, **86**, 733 (1930).
7. Hofmann, K. A., *Ann. Chem.*, **312**, 1 (1900).

TWO FACTORS INFLUENCING THE SERUM CALCIUM AND INORGANIC PHOSPHATE OF THE RABBIT

I. THE INFLUENCE OF DIET
II. DIURNAL VARIATION

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The extensive use of the rabbit as an experimental animal, and the wide range of the normal level of serum calcium and inorganic phosphate as found by various workers and ourselves, have led us to investigate the usefulness of this animal for calcium and phosphorus investigations.

A careful search of the available literature revealed several possible explanations; e.g., differences due to sex, seasonal variation, age, environment, hemorrhage, and diet. As our experiments will show, the differences attributed to sex, seasonal variation, age, environment, and hemorrhage are of no great significance compared to those due to diet.

Technique

The ear of the rabbit from which blood was to be drawn was shaved and cleaned with ether. The superficial vessels were dilated by warming near an ordinary electric lamp for 2 or 3 minutes, then rubbed gently with benzene or xylene. The benzene or xylene was carefully removed by wiping. A diagonal incision was made in one of the marginal veins and the blood allowed to drop directly into a clean, dry test-tube. About 5 cc. of blood were collected.

The method used for inorganic phosphorus determinations was that of Briggs (1). The sulfite and hydroquinone solutions were made up together. 1 cc. of serum was used for each determination.

The method of calcium estimation used was that of Kramer and Tisdall as modified by Clark and Collip (2). 1 cc. of serum was used. After addition of the oxalate the serum was allowed to stand overnight to allow sufficient time for complete precipitation. The precipitate was washed once with 2 cc. of 2 per cent ammonia water. The first pink color lasting for 30 seconds with vigorous shaking was taken as the end-point in the permanganate titration. The titrations were made against time, 90 seconds being allowed as the maximum.

<div align="center">EXPERIMENTAL</div>

Table I shows the variations in the normal levels of serum calcium and inorganic phosphorus as found by some other workers.

<div align="center">TABLE I</div>

Variations in Normal Serum Calcium and Inorganic Phosphorus
The results are expressed in mg. per cent.

Authors	Calcium			Phosphorus		
	High	Low	Mean	High	Low	Mean
Harris and Stewart (3)	13.0	9.5	11.1	5.7	4.0	4.6
Culhane (4)	17.1	11.9	14.4			
Brown (5, 6)	17.5	13.5	*	7.7	2.5	*
Mirvish and Bosman (7)	18.0	11.5	15.2			

* The mean value is not given.

Blood samples taken from our own normal animals at various times exhibit a vastly divergent range, which is quite compatible with the values in Table I. In the case of calcium, values as low as 7.6 and as high as 22.0 mg. per cent were found. The most common percentage, however, was between 9.5 and 18.5 mg. The extreme limits of inorganic phosphorus were 1.2 to 9.0 mg. per cent, the more usual being 2.2 to 6.9 mg. per cent. Fig. 1 shows the variations in calcium and phosphorus in two normal female chinchilla rabbits under observation for 2 months. These animals received the ordinary stock diet of cabbage, bran, and oats. The figures for Rabbit A-1 showed a variation from 10.0 to 16.6 mg. per cent for calcium, and from 1.4 to 7.5 mg. per cent for phosphorus, with a mean value of 13.7 and 3.8 mg. per cent

respectively.   For Rabbit A-2 the values ranged between 9.4 and
15.8 mg. per cent for calcium, and between 2.5 and 8.5 mg. per
cent for phosphorus, the mean values being 13.3 and 5.8 mg.
per cent respectively.   Fig. 1 also shows definitely that a rise in
calcium is accompanied by a simultaneous fall in inorganic phos-
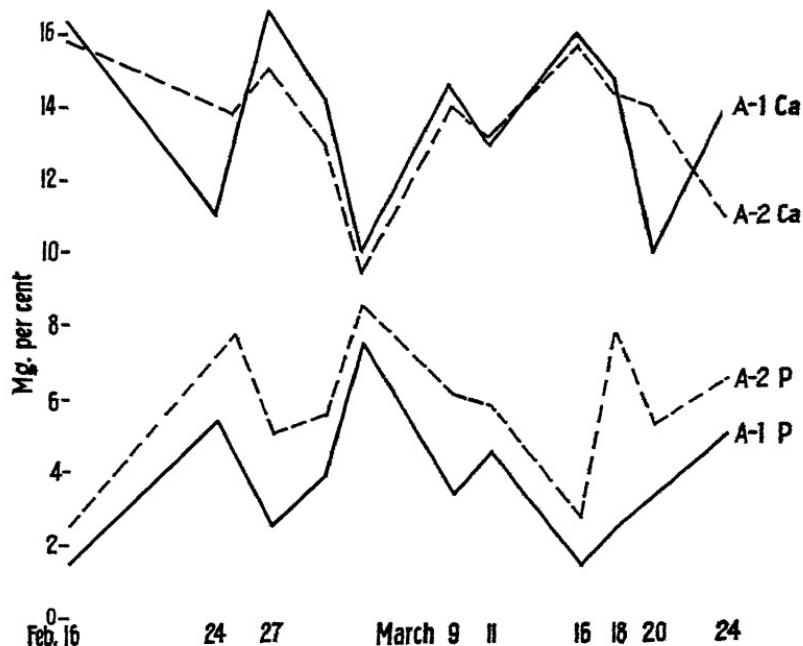
Fig. 1. Random variations in the level of serum Ca and P in two normal
female chinchilla rabbits (Rabbits A-1 and A-2) during an interval of 6
weeks. The similarity between the changes shown by the two animals
points to some external cause affecting them both. The composition of
the diet varied in an uncontrolled manner from day to day, but was the
same for the two animals on any one day.

phorus, and *vice versa*.   This tendency to an inverse variation has
also been pointed out by Brown (5).

I. Influence of Diet

The most obvious physiological explanation of fluctuations of
serum calcium and phosphorus is diet.

Culhane (8), states that "the consumption of cabbage pro-
duces a definite, though temporary, rise in serum calcium of rab-

bits." At the 4th hour after cabbage feeding there was an average rise of 1.2 mg. per cent from the initial value. At the 6th hour the calcium returned to normal. Although this is not a dramatic change in itself, it is definitely significant. At the end of the 2nd hour, however, there is a fall of 0.98 mg. per cent, which is almost
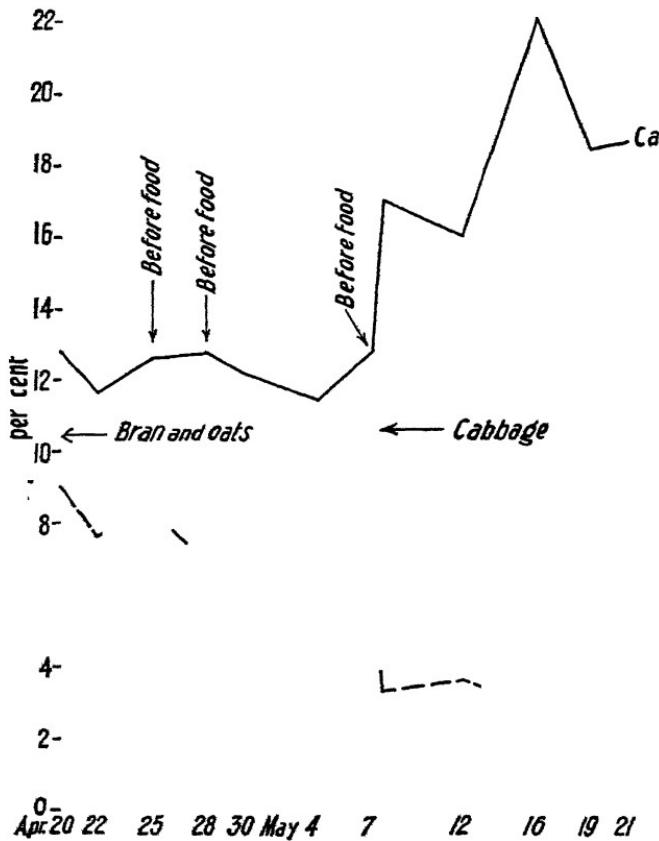
Fig. 2. Effect of a change from a diet of bran and oats (April 20 to May 7) to a diet of cabbage (May 7 to 21) on the serum Ca and P levels of a female chinchilla rabbit.

as great a change as the rise of 1.22 mg. per cent at the 4th hour. For this Culhane offers no explanation.

Kapsinow and Underhill (9) conclude that as a result of their study "it is impossible to state that cabbage has a definite calcium-raising substance."

To show the effect of diet we have subjected a series of rabbits to a systematic and simple dietary.   The first group of rabbits (see Fig. 1) was fed for 2 months on the ordinary stock diet of mixed bran and oats and cabbage.   During this period they showed a mean value of 13.5 mg. per cent for calcium and 4.8 mg. per cent for inorganic phosphorus, with variations in calcium from
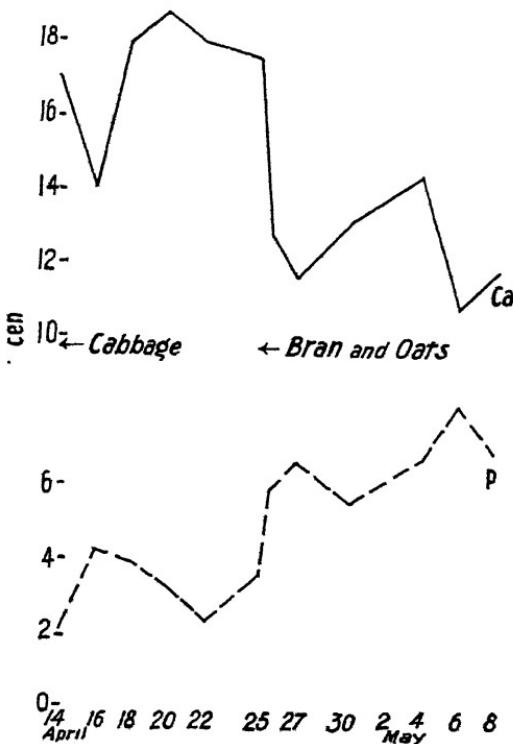
Fig. 3. Effect of a change from a diet of cabbage (April 14 to 25) to a diet of bran and oats (April 25 to May 8) on the serum Ca and P levels of a female chinchilla rabbit.

9.4 to 16.0 and in phosphorus from 1.4 to 8.5 mg. per cent.   Although the average values for calcium and phosphorus concentrations are approximately those given as the normal by others, the wide fluctuations from this level have still to be explained.   We decided that this was due to the fact that on some days the rabbits received only bran and oats, and on other days only cabbage.

We then segregated the animals, feeding one group solely upon cabbage and the other upon a mixture of bran and oats.[1]

Figs. 2 and 3 show the results from a typical rabbit of each group. Rabbits fed on bran and oats exhibit a tendency towards a low calcium and a high phosphorus level, but even on this
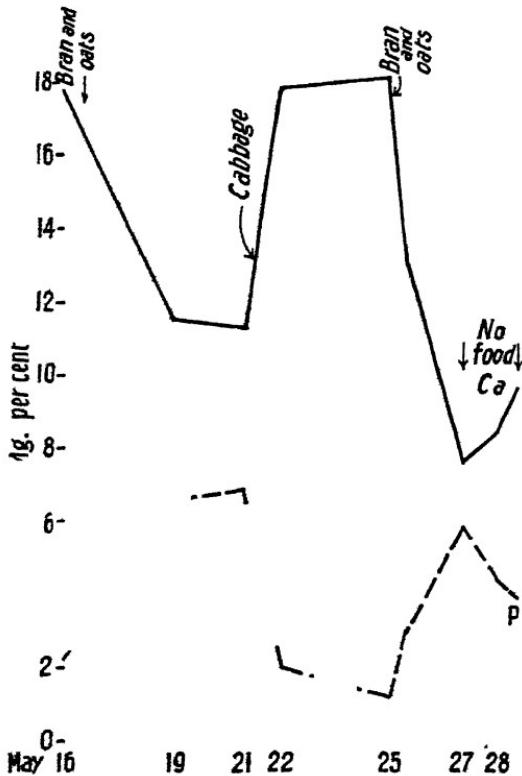
FIG. 4. Rapid alternations between a cabbage diet and one of bran and oats. Note the rise in serum Ca level when food is withheld after a period of bran and oats feeding.

constant diet there is still a slight fluctuation. Rabbits fed on cabbage exhibit a tendency towards a high calcium and a low phosphorus level, with a fluctuation even more marked than in those fed on bran and oats. Reversing the diets brought about

[1] We are aware that bran and oats are not a perfect diet, but we observed no ill effects during the course of our experiments.

the expected effects; *i.e.*, the low calcium level due to bran and oats was raised, while the high level due to cabbage fell. The inorganic phosphorus also shows a correspondingly marked change. Fig. 2 shows that when the diet of bran and oats is changed to one of cabbage there is a marked rise in 6 hours of 4.2 mg. per cent in calcium, and a fall of 3.3 mg. per cent in phosphorus. Continued feeding of cabbage produced a further rise in calcium and
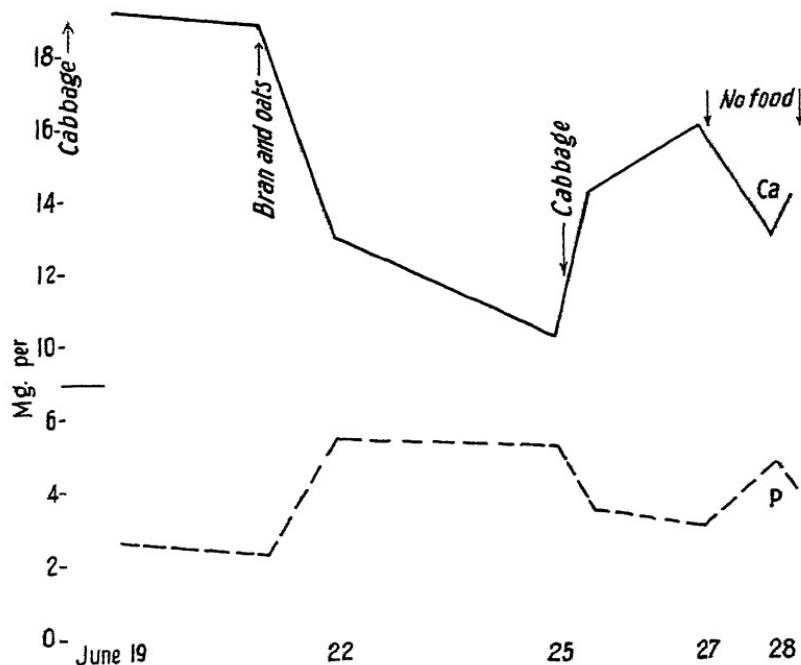
FIG. 5. A further example of frequent alternations between a cabbage diet and one of bran and oats. Note that withholding of food after a period of cabbage feeding results in a fall in serum Ca level.

fall in phosphorus concentrations. 12.2 and 7.5 mg. per cent were the averages for the serum calcium and phosphorus during the interval of bran feeding. The ranges during this period were 11.4 to 12.8 and 6.6 to 9.0 mg. per cent respectively. During the period of feeding cabbage the average values for calcium and phosphorus were 18.3 and 3.0 mg. per cent respectively, with a range of 16.0 to 21.8 mg. per cent for calcium and of 2.4 to 3.6 mg. per cent for phosphorus.

We attach significance to the fact that the blood samples taken 12 hours after the last feed of bran and oats show a fairly constant calcium level. The three instances cited in Fig. 2 show values of 12.6, 12.8, and 12.8 mg. per cent.

Figs. 4 and 5 show still more dramatic responses to changes of diet. Feeding cabbage definitely produces a high concentration of serum calcium and a fall in that of inorganic phosphate, while a change to bran and oats produces the reverse effect. Withholding food for 12 to 24 hours from animals previously fed upon bran and oats results in a rise in calcium concentration and a fall in phosphorus. This indicates that the phosphorus level had been exceeded and the calcium level correspondingly depressed, and that the true normals are now being established. In our opinion bran and oats also have a specific effect on serum calcium and phosphorus, but in the opposite direction to that of cabbage. When food is withheld from animals previously fed on cabbage, there is a definite fall in calcium concentration with a corresponding rise in phosphorus. This indicates that in this case, also. during fasting the serum calcium and phosphorus tend to establish their normal equilibrium.

DISCUSSION

In our opinion the essential factor causing fluctuation in the concentration of serum calcium and phosphorus is diet. In an endeavor to ascertain the direct cause of these changes we have had to eliminate, as insignificant, many factors put forward in previous works. As we have shown in the above experiments the magnitude of the immediate fluctuations caused by diet overshadows changes attributed to the other factors.

The question of the advisability of using rabbits for experiments on calcium and phosphorus in the blood serum has to be considered in the light of the above facts. In an experiment which is expected to raise the blood calcium, we suggest that the animal should be kept on a diet which will give a low initial calcium value; e.g., bran and oats. It would be essential to keep the animal on such a diet for 2 or 3 days and then to fast it for 18 hours prior to, and during the experiment. This of course is possible only for short term experiments. For experiments covering a period of more than 2 days this procedure would be incompatible with

normal body metabolism, and therefore we consider the rabbit an unsuitable animal for such experiments. The withholding of food for 18 hours prior to the experiment is necessary to allow the calcium and phosphorus to establish an equilibrium. As we have shown above, a diet of bran and oats raises the phosphorus to such a high level that this may be the influencing factor in keeping the calcium out of the blood stream. Although we have not investigated the fate of the calcium on this diet, it is reasonable to assume that the calcium is stored in the body tissues, as is indicated by the slight but consistent rise occurring after 12 to 24 hours fasting (*cf.* Figs 2 and 4).

II. *Diurnal Variation*

In the first part of this paper we have shown that diet is the principal factor causing fluctuations in the normal serum calcium
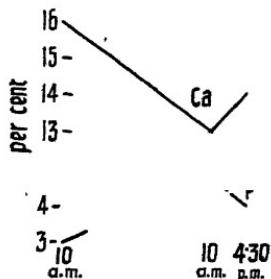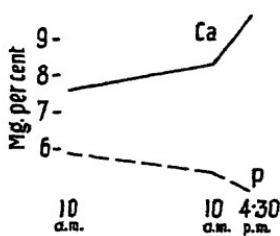
FIG. 6, *a*   FIG. 6, *b*

FIGS. 6, *a* AND 6, *b*. Illustrating the diurnal variation in Ca and P levels of the serum of fasting rabbits. For details see text.

and phosphorus level of rabbits. After having arrived at a means of establishing a normal level for serum calcium and phosphorus by withholding food for 12 to 24 hours, and thus eliminating the effects due to diet, we decided to investigate the question of diurnal variation.

<div align="center">EXPERIMENTAL</div>

The technique used was the same as in the preceding section.

An adult female chinchilla rabbit, fed previously on cabbage (Fig. 6, *a*), shows a fall in calcium after 24 hours fasting, with a subsequent rise 6 hours later, while the inorganic phosphorus shows a rise and subsequent fall. Fig. 6, *b* (an adult female chinchilla

rabbit, previously fed on bran and oats) shows after 24 hours
fasting a slight rise in calcium and a fall in inorganic phosphorus.
6 hours later there was a further slight rise and fall in calcium and
phosphorus respectively.

Such a subsequent rise has been attributed by Charles (10) to
the effect of a second bleeding.   On the other hand, Kapsinow
and Underhill (9) state that there is a fall in calcium due to bleed-
ing.   From the result of our experiments, we are of the same opin-
ion as Culhane (8) and Mirvish and Bosman (7), who report that
repeated withdrawal of blood from a rabbit has no connection
with the blood calcium level.

The second slight rise in calcium and fall in phosphorus is quite
significant of a *diurnal variation,* rather than the effect of bleed-

TABLE II

Diurnal Variation of Calcium and Phosphorus Levels in Fasting Rabbits

The results are expressed in mg. per cent.

Rabbit No.	Experiment 1				Experiment 2			
	10 a.m.		10 p.m.		10 p.m.		10 a.m.	
	Ca	P	Ca	P	Ca	P	Ca	P
D-1	13.0	4.8	14.0	4.0	13.0	4.6	11.6	5.4
D-2	13.4	5.0	14.1	4.4	12.2	7.5	11.6	7.5
D-3	8.4	4.4	9.6	3.9	14.3	4.3	13.0	6.5
D-4	12.4	7.2	12.4	6.3	14.2	3.5	13.0	4.8
D-5	11.6	7.5	12.6	7.0	13.7	4.7	12.3	5.2

ing.   To check this effect, food was withheld for 12 to 24 hours
and a sample of blood was taken in the morning and another at
night 12 hours later.[2]   Experiment 1 in Table II shows the results.
The evening samples were higher in calcium and lower in phos-
phorus concentration.   Experiment 2, Table II, shows the result
of reversing this procedure by taking the first sample at night
and the second the following morning, 12 hours later.   In this
case there was a fall in calcium and a corresponding rise in phos-
phorus in the second sample.

It is obvious from these results that these variations are not due
to hemorrhage, and the change depends on the time of day when
the blood is withdrawn.

[2] When food was withheld the animals were supplied with water.

CONCLUSIONS

1. Cabbage fed to rabbits causes a marked rise in the serum calcium, and a simultaneous fall in the inorganic phosphorus concentration.

2. Bran and oats fed to rabbits cause a marked rise in the inorganic phosphorus of serum, with a simultaneous fall in calcium.

3. A rise in calcium is accompanied by a fall in inorganic phosphorus, and *vice versa*.

4. There is a definite *diurnal variation* in the serum calcium and phosphorus levels of the rabbit. The evening calcium level is 1.0 to 1.5 mg. per cent higher than the morning sample. The change in phosphorus is of about the same magnitude.

5. In view of this *diurnal variation,* a rise or fall of less than 1.5 mg. per cent in serum calcium and phosphorus is of no significance.

6. Rabbits are not suitable animals for experiments in which changes of calcium and phosphorus in the blood serum are effected, if such experiments are to cover a period of more than 2 days.

BIBLIOGRAPHY

1. Briggs, A. P., *J. Biol. Chem.*, **53,** 13 (1922).
2. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, **63,** 461 (1925).
3. Harris, L. J., and Stewart, C. P., *Biochem. J.*, **23,** 206 (1929).
4. Culhane, K., *Biochem. J.*, **21,** 1015 (1927).
5. Brown, W. H., *J. Exp. Med.*, **47,** 539, 563 (1928).
6. Brown, W. H., *J. Exp. Med.*, **47,** 637 (1928).
7. Mirvish, L., and Bosman, L. P., *Quart. J. Exp. Physiol.*, **18,** 14 (1927).
8. Culhane, K., *J. Biol. Chem.*, **86,** 113 (1930).
9. Kapsinow, R., and Underhill, F. P., *J. Biol. Chem.*, **82,** 377 (1929).
10. Charles, E., *Quart. J. Exp. Physiol.*, **21,** 81 (1931).

STUDIES IN POLYMERIZATION AND CONDENSATION

VII. POLYMERIZATION OF THE α-HYDROXYALDEHYDES

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The α-hydroxyaldehydes have attracted the attention of chemists by reason of their chemical resemblance to sugars. Like sugars, they are capable of forming cycloacetals but, unlike sugars, they readily polymerize and the polymeric forms, more accurately speaking the dimeric forms, are readily reconverted into the monomeric forms. Their capacity for forming cycloacetals suggests the possibility of a cyclic structure for the free hydroxyaldehydes; on the other hand, the readiness with which they polymerize is analogous to that of simple aldehydes and therefore suggests the other, namely the aldehydic structure.

A knowledge of the chemical structure of those hydroxyaldehydes which differ from one another by the distance of the hydroxyl from the carbonyl group may be of assistance in formulating a conclusion regarding the ring structure of non-substituted sugars inasmuch as direct methods for solving this problem as yet are not available.

Several γ-hydroxyaldehydes (4-hydroxyaldehydes) have been prepared by Helferich and his students,[1] and for these substances he assumes the cyclic structure, although only in the case of phenylbutyraldehyde was this conclusion substantiated by a molecular weight determination. Aldol, the representative of β-hydroxyaldehydes, likewise readily undergoes polymerization.

Thus, existing data seem to indicate that the α- and β-hydroxyaldehydes, similarly to unsubstituted aldehydes, readily undergo polymerization, whereas the γ-hydroxyaldehydes seem to behave similarly to sugars passing readily into the cyclic form.

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[1] Helferich, B., and coworkers, *Ber. chem. Ges.*, **52**, 1123, 1800 (1919); **54**, 930, 2640 (1921); **56**, 2088 (1923).

In view of the fact that we were in need of α-hydroxyaldehydes for other investigations, we took the occasion to follow in some detail their tendency towards polymerization. Of the members of the normal series of α-hydroxyaldehydes, only the α-hydroxypropionic and α-hydroxyheptylic[2] aldehydes have been made the subject of careful study in recent years. In these cases the aldehydes, in homogeneous state, readily polymerized into the dimeric form. The degree of polymerization was established by molecular weight determination.

The α-hydroxyaldehydes used in the present investigation were prepared from unsaturated carbinols by the reduction of the ozonides in glacial acetic acid solution by means of zinc dust[3] according to the method of Harries,[4] a method successfully used for the same purpose in this laboratory on a previous occasion and prior to that by Helferich and coworkers for the preparation of γ-hydroxyaldehydes. We may add here that as a general method for the preparation of α-hydroxyaldehydes it is to be preferred to the method of Franke which proceeds by way of the α-bromoaldehydes.[5] The following α-hydroxyaldehydes have been prepared by the ozone method.

1. α-Hydroxypropionic aldehyde from methylvinylcarbinol
2. α-Hydroxy-*n*-butyric aldehyde from ethylvinylcarbinol
3. α-Hydroxy-*n*-valeric aldehyde from propylvinylcarbinol
4. α-Hydroxy-*n*-hexylic aldehyde from octene-2-ol-4
5. α-Hydroxy-*n*-heptylic aldehyde from amylvinylcarbinol

Spontaneous Polymerization of Homogeneous α-Hydroxyaldehydes
—The α-hydroxyaldehydes here described are all very mobile liquids at room temperature when freshly distilled. As soon as the receiving flask containing the distillate is removed from the cooling mixture, the temperature of the liquid begins to rise, attaining a value of about 10° above room temperature within 1 hour. This

[2] Dworzak, R., and Pfifferling, P., *Monatsh. Chem.*, 48, 251 (1927). Dworzak, R., and Prodinger, W., *Monatsh. Chem.*, 50, 459 (1928). Fischer, H. O., *Ber. chem. Ges.*, 62, 854 (1929).

[3] We wish to express our appreciation to the Sullivan Mining Company for the excellent zinc dust which they generously supplied for this experiment.

[4] Harries, C., *Ann. Chem.*, 410, 5 (1915).

[5] Franke, A., *Monatsh. Chem.*, 21, 205, 210 (1900).

rise of temperature is accompanied by a rapid increase in both the viscosity and the refractive index of the liquid and in most cases the substance crystallizes gradually. The freshly distilled liquid gives a positive Schiff's test and shows a very definite absorption band in the ultra-violet region (2980 Å) which is characteristic for the aldehyde group (see Fig. 1).

The crystalline material shows a lagging melting point, apparently due to partial depolymerization on heating. This assump-
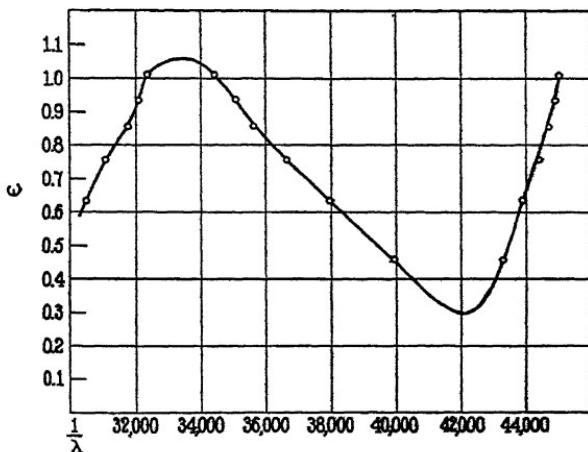
Fig. 1. Ultra-violet absorption spectrum of α-hydroxyhexylaldehyde. A Hilger sector spectrophotometer was used. All measurements were made in absolute ethyl alcohol. The logarithms of the molecular extinction coefficients are plotted against the reciprocals of the wave-lengths. (We wish to express our thanks to Dr. Alexandre Rothen for making the absorption measurements.)

tion is justified by the observation that the material removed from the melting point tubes gives a positive Schiff's test, whereas the original crystalline material does not. The crystalline material does not possess the absorption band in the ultra-violet region characteristic of the monomeric form. Determination of the molecular weight of the crystalline form shows it to be dimolecular.

No definite statement can be made regarding the structure of the dimeric forms. Two possible structures (I and II) have been discussed in the past. To these, a third (III) can now be added which

would bring the process of polymerization of the hydroxyaldehydes in line with that of unsaturated compounds.   There are several reasons which favor the third possibility: firstly, the occurrence of this form of condensation in the case of unsubstituted aldehydes; secondly, the fact that the trehalose type of condensation is the
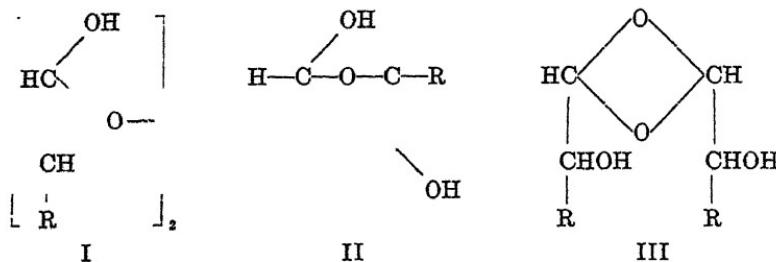
one which is easiest to accomplish in the case of sugars; thirdly, the easy depolymerization of the dimeric forms; and finally, the fact that in the case of the optically active aldehydes the original rotation is restored after depolymerization.

<div align="center">EXPERIMENTAL</div>

The α-hydroxyaldehydes described were obtained by the ozonization of unsaturated carbinols, a process generally carried out in the following way.   15 gm. of the unsaturated carbinol were dissolved in 25 cc. of glacial acetic acid and a dry stream of ozonized oxygen was passed through the solution until a test portion no longer decolorized a solution of bromine in glacial acetic acid. The main portion was now diluted with about 4 times its volume of dry ether and the ozonide decomposed with a little water in the presence of zinc.[6]   This operation was carried out under a reflux condenser with mechanical stirring.   The filtered solution was almost neutralized with finely pulverized potassium bicarbonate and neutralization was completed by means of a solution of potassium carbonate.   The ether solution was dried over sodium sulfate, filtered, and the ether distilled off.   The residue was fractionated under reduced pressure.

Lactic Aldehyde—The polymerization of this aldehyde had been

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[6] Helferich, B., *Ber. chem. Ges.*, **52**, 1123 (1919).

studied by Wohl[7] and by Dworzak and Prodinger.[8] Only the early changes of the freshly prepared solution are reported here.

This α-hydroxypropionic aldehyde was prepared by ozonization of pentene-2-ol-4. The reaction mixture was worked up in a manner similar to that described above, and on distillation, a fraction was collected which boiled between 70–100° at 16 to 20 mm. The vapors were colorless, but the distillate received in a flask cooled by carbon dioxide-alcohol was lemon colored. On warming to room temperature, this material became a very mobile liquid which evolved heat and exhibited an increase in viscosity. At the same time the refractive index rose from 1.437 to 1.565 at 25° in less than an hour.

α-Hydroxybutyric Aldehyde—For this aldehyde also the early changes of the monomeric form are described.

It was obtained on ozonization of ethylvinylcarbinol. On distillation, a main fraction was obtained which distilled between 35–60° at 30 mm. It had the following composition.

4.442 mg. substance: 9.018 mg. CO_2 and 3.691 mg. H_2O.
$C_4H_8O_2$ (88.06). Calculated. C 54.51, H 9.16
Found. " 55.36, " 9.30

The substance evolved heat on standing and became viscous, reduced Fehling's solution in the cold, and gave Schiff's test.

α-Hydroxyvaleric Aldehyde—Dextro-α-hydroxyamylaldehyde has been obtained by Levene and Haller[9] by ozonization. The hydroxyaldehyde was not isolated by these authors, but transformed directly into the corresponding pentanediol by means of sodium amalgam. The racemic α-hydroxyvaleric aldehyde has now been obtained, both in the monomeric and the dimeric forms, by the procedure mentioned above. The ozonide in solution was worked up in a manner similar to that described above. On distillation, a fraction was collected which boiled at 65–85° at 15 mm. The fraction which distilled at 70° at 15 mm. was analyzed. The substance had the following composition.

2.911 mg. substance: 6.333 mg. CO_2 and 2.585 mg. H_2O.
$C_5H_{10}O_2$ (102.1). Calculated. C 58.77, H 9.87
Found. " 59.32, " 9.94

[7] Wohl, A., *Ber. chem. Ges.*, **41**, 3599 (1918).

[8] Dworzak, R., and Prodinger, W., *Monatsh. Chem.*, **50**, 459 (1928).

[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 579 (1929).

This fraction, as well as some of the fractions of a less definite boiling point, evolved heat spontaneously and showed both the physical and the chemical changes described above. After three recrystallizations from acetone the crystalline substance melted at 145°, the temperature of the bath being raised quickly. Further recrystallizations may cause a still further increase in the melting point.

α-Hydroxyhexylic Aldehyde (Hexanal-1-ol-2)—Dextro- and levo-α-hydroxyhexylaldehydes have been obtained by Levene and Haller[9] by ozonization of levo-octene-2-ol-4 and dextro-heptene-1-ol-3 respectively. Both the monomeric and dimeric racemic α-hydroxyhexylaldehydes have now been prepared from inactive octene-2-ol-4 in the same way. The latter substance was obtained in a 70 per cent yield, from butylmagnesium bromide and crotonic aldehyde. The ozonization and isolation were carried out as described above. On distillation a fraction was obtained which boiled at approximately 60° at 1 to 2 mm., and showed all the properties described above.

The distillate, which had crystallized, was at first recrystallized from hot alcohol but, as it was found that hot acetone is more suitable for recrystallization, the substance was thus recrystallized six times, the melting point on rapid heating being about 150°. The crystalline form is insoluble in cold water; it had the following composition.

4.105 mg. substance : 9.380 mg. CO_2 and 3.850 mg. H_2O.
$C_6H_{12}O_2$ (116.1).   Calculated.   C 62.02, H 10.42
Found.   " 62.31, " 10.49

Determination of Molecular Weight of Crystalline α-Hydroxyhexylic Aldehyde by Method of Menzies and Wright—0.3845 gm. of substance dissolved in 32 cc. of absolute alcohol (b.p. 78.4° at 758 mm.) gave 15 mm. elevation on a differential thermometer.

Mol. wt., dimeric. $(C_6H_{12}O_2)_2$.   Calculated.   232
Found.   241

Conversion of Dimeric α-Hydroxyhexylic Aldehyde into the Monomeric Form—Recrystallized dimeric α-hydroxyhexylaldehyde was distilled from a double neck distilling flask at approximately 0.5 mm. pressure, the receiving flask being cooled with a mixture of

solid carbon dioxide and alcohol. The bath temperature was raised slightly above the melting point of the dimeric substance, and the distillation continued at a somewhat lower bath temperature. The distillate was a solid, of crystalline appearance, which started to melt rapidly on removal from the cooling mixture and turned into a very mobile liquid. A sample of this material on the refractometer (25°) exhibited a rapid increase of the refractive index. At the same time the temperature of another sample rose above room temperature. A sample of the freshly distilled substance showed absorption in the ultra-violet spectrum, the maximum occurring at a wave-length of 2980 Å. After a few more minutes, crystallization set in, and the following morning the entire substance was again a solid mass of crystals.

Dextro-α-Hydroxyheptylic Aldehyde—The dextro-α-hydroxyheptylaldehyde was obtained by ozonization of levo-amylvinylcarbinol[9] and the product was isolated in the usual manner. On fractionation, the hydroxyaldehyde distilled at 70–75° at approximately 3 mm. pressure, the receiver being cooled with a mixture of solid carbon dioxide and alcohol. The distillate was of crystalline appearance and possessed all the general properties of an α-hydroxyaldehyde. Furthermore, due to its asymmetric carbon atom, it showed a pronounced increase in optical rotation during the process of autopolymerization. The rotation of the substance (homogeneous) changed from $\alpha_D^{26} = +5°$ to $\alpha_D^{26} = +27°$. It had the following composition.

3.570 mg. substance: 8.395 mg. CO_2 and 3.492 mg. H_2O.

$C_7H_{14}O_2$ (130.1).   Calculated.   C 64.57, H 10.84
                           Found.   " 64.12, " 10.94

On prolonged standing the viscous material gradually crystallized into a substance which after one recrystallization from acetone melted at 120°.

Levo-α-Hydroxyheptylic Aldehyde—This levorotatory hydroxyaldehyde was prepared by ozonization of dextro-amylvinylcarbinol ($\alpha_D^{24} = +14.3°$).[10] On fractionation a portion was obtained which distilled at 109–111° at 25 mm., the receiver being cooled in an ice-salt mixture. The distillate crystallized in needles which, however, melted rapidly on removal from the cooling mixture.

[10] Levene, P. A., and Walti, A., unpublished data.

The substance had the following composition.

5.210 mg. substance: 12.200 mg. CO_2 and 4.960 mg. H_2O.
$C_7H_{14}O_2$ (130.1). Calculated. C 64.57, H 10.84
Found. " 63.85, " 10.65

On standing, the aldehyde polymerized into a viscous mass having a rotation of $\alpha_D^{26} = -24°$ (homogeneous). On redistillation the aldehyde was obtained again in the monomeric form, with the rotation of $\alpha_D^{26} = -4.1°$ (homogeneous), which this time did not undergo the usual rapid change in viscosity or in rotation. This phenomenon may be attributed to a slight impurity, perhaps an oxidation product of the aldehyde.

PHYTOCHEMICAL REDUCTIONS

THE CONFIGURATIONS OF GLYCOLS OBTAINED BY REDUCTION WITH FERMENTING YEAST

BY P. A. LEVENE AND A. WALTI

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

The preparation of optically active glycols by means of fermenting yeast was accomplished first by Neuberg and his coworkers.[1] The method has been used extensively in this laboratory with success.[2] The question naturally arose as to the configurational relationship of the various glycols prepared in this manner. Glycols of two types have been thus prepared in the past; namely, the 1,2 diols and one 1,3 diol. The former have been prepared from 1-hydroxyketones-(2) and the latter from butanol-(3)-al-(1)-$CH_3CHOHCH_2CHO$. In the first case the ketonic carbonyl group was reduced to an optically active secondary carbinol group; in the second case, the carbonyl group was reduced to a primary carbinol group and the resulting glycol was optically active.

All hydroxyketones of the type of propanol-(1)-one-(2) yielded glycols of identical configuration; namely,

$$
\begin{array}{c}
\text{OH} \\
| \\
\text{(HO)H}_2\text{C} - \text{C} - \text{R} \\
| \\
\text{H}
\end{array}
$$
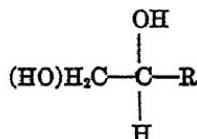
[1] Färber, E., Nord, F. F., and Neuberg, C., *Biochem. Z.*, **112**, 313 (1920). Neuberg, C., and Kerb, E., *Biochem. Z.*, **92**, 96 (1918).

[2] Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, **71**, 465 (1926–27).

The aldol gave a glycol of the following configuration

$$
\begin{array}{c}
\text{OH} \\
| \\
\text{CH}_3\text{---}\text{C}\text{---}\text{CH}_2\text{CH}_2\text{OH} \\
| \\
\text{H}
\end{array}
$$
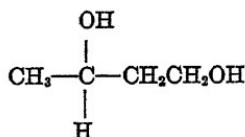
If the respective positions of the two primary carbinol groups are taken as a basis for the correlation of the two substances expressed by the above two formulas, they may be regarded as enantiomorphous.

It seemed of interest to determine whether all hydroxyketones would follow the first mode of reduction and all α-hydroxy-aldehydes the second. With the view of answering this question α-hydroxyaldehydes and 4-hydroxybutanone-(2) were subjected to the action of fermenting yeast.

The reduction of the α-hydroxyaldehydes was accomplished in every instance. Although the yields of the glycols were generally quite good, the magnitude of the optical activity was either doubtful or very small. Only in the case of α-hydroxyamylaldehyde was the optical activity of the substance established by the preparation of the optically active diphenylurethane. The glycol prepared in this manner had the same configuration as in the series of the hydroxy-ketones described above; namely, the one given by the following formula

$$
\begin{array}{c}
\text{OH} \\
| \\
(\text{HO})\text{H}_2\text{C}\text{---}\text{C}\text{---}\text{R} \\
| \\
\text{H}
\end{array}
$$
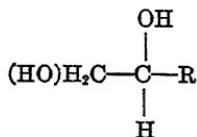
The reduction of 4-hydroxybutanone-(2) yielded a glycol of the configuration

$$
\begin{array}{c}
\text{OH} \\
| \\
(\text{HO})\text{H}_2\text{C}\cdot\text{H}_2\text{C}\text{---}\text{C}\text{---}\text{R} \\
| \\
\text{H}
\end{array}
$$
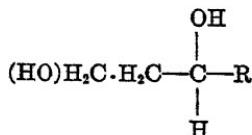
To sum up, the reactions proceeded in the following way.

$$CH_2OH \cdot CH_2COCH_3 \qquad\qquad CH_2(OH)COR$$

$$
\begin{array}{cc}
\overset{\displaystyle OH}{\big\downarrow} & \overset{\displaystyle OH}{\big|} \\
CH_2OHCH_2\!-\!\underset{\underset{\displaystyle I}{\textstyle H}}{\overset{\displaystyle |}{C}}\!-\!CH_3 & CH_2OH\!-\!\underset{\underset{\displaystyle II}{\textstyle H}}{\overset{\displaystyle |}{\dot C}}\!-\!R
\end{array}
$$

$$CHOCH(OH)R \qquad\qquad CH_3CH(OH)CH_2CHO$$

$$
\begin{array}{cc}
\overset{\displaystyle OH}{\big\downarrow} & \overset{\displaystyle OH}{\big|} \\
CH_2OH\!-\!\underset{\underset{\displaystyle III}{\textstyle H}}{\overset{\displaystyle |}{C}}\!-\!R & CH_3\!-\!\underset{\underset{\displaystyle IV}{\textstyle H}}{\overset{\displaystyle |}{\dot C}}\!-\!CH_2CH_2OH
\end{array}
$$
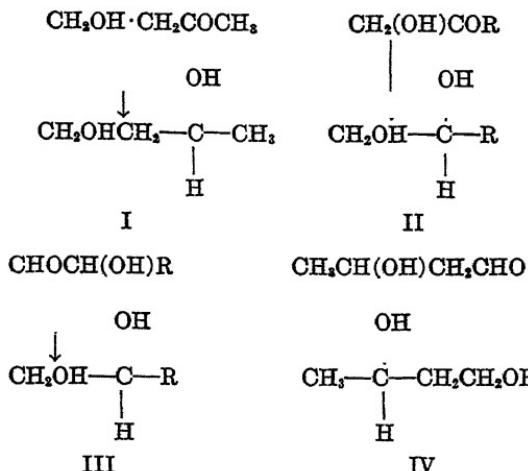
Before concluding, we wish to add that, by fermentation, Friedmann[3] recently reduced acetoacetic acid to dextro-β-hydroxybutyric acid having the following configuration.

$$CH_3\!-\!\underset{\textstyle H}{\overset{\textstyle OH}{\underset{|}{\overset{|}{C}}}}\!-\!CH_2\!-\!COOH$$
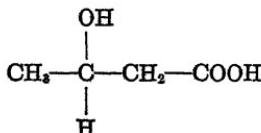
This β-hydroxybutyric acid (ester) can be reduced to a glycol of configuration identical with that obtained on reduction of the aldol.

SUMMARY

1. It has been found that hydroxyketones of the series of propanol-(1)-one-(2) are reduced by fermenting yeast to glycols of the type

$$CH_2OH\!-\!\underset{\textstyle H}{\overset{\textstyle OH}{\underset{|}{\overset{|}{C}}}}\!-\!R$$
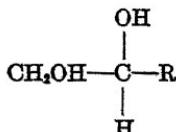
[3] Friedmann, E., *Naturwissenschaften*, **19**, 400 (1931).

2. The α-hydroxyaldehydes are not so readily reduced as the hydroxyketones but, when reduced, form optically active glycols of the same configuration as those derived from the hydroxyketones.

3. Two isomeric substances, aldol and 4-hydroxybutanone-(2), form glycols of opposite configurations.

4. Acetoacetic acid is reduced to an acid having the same configuration as the glycol obtained from aldol.

5. From the fact mentioned in (3), it follows that the optically active glycols produced on fermentation are not the result of the fermentability of one of the enantiomorphs, but are the result of a true asymmetric reduction.

<center>EXPERIMENTAL</center>

General Procedure of Reduction—10 parts by weight of sugar were dissolved in 100 parts of water, and 10 parts of bakers' yeast[4] were added. When the mixture was fermenting vigorously, a solution of the hydroxyaldehyde (1 part) in its own volume of alcohol was added drop by drop. About 50 gm. of yeast were added each day for the next 3 days. When a filtered sample of the mixture ceased to reduce Fehling's solution, the fermentation mixture was treated with charcoal, filtered, and evaporated under reduced pressure. The syrupy residue was treated with absolute alcohol and a little anhydrous ether, the precipitate removed, and the solution again concentrated. The residue was treated once more with absolute alcohol and dry ether, the alcohol-ether solution again concentrated, and the residue fractionally distilled. The yield of the glycol was 50 to 60 per cent of the hydroxyaldehyde used.

Hexanediol-(1,2) from α-Hydroxyhexylaldehyde—5 gm. of α-hydroxyhexylaldehyde[5] dissolved in 10 cc. of absolute alcohol were added to the fermenting sugar solution previously described. In this case the mixture was well stirred for 4 days with addition of yeast each day. After the mixture had been treated as described above, 2.5 gm. of a crude hexanediol-(1, 2) were obtained. It was refractionated, the main portion distilling at 111° at 12 mm. The substance had the following composition.

3.520 mg. substance: 7.892 mg. CO_2 and 3.850 mg. H_2O.

$C_6H_{14}O_2$ (118.1).   Calculated.   C 60.97, H 11.94
Found.   " 61.14. " 12.23

The specific rotation of the glycol was as follows:

$$[\alpha]_D^n = \frac{+0.25° \times 100}{2 \times 21.55} = +0.58° \text{ (in absolute alcohol)}$$

It is not certain that activity was not due to an impurity, inasmuch as the redistilled available material was too small in quantity to permit of the preparation of a urethane.

Pentanediol-(1,2) from α-Hydroxyamylaldehyde—14 gm. of pentanal-1-ol-2 dissolved in absolute alcohol were subjected to phytochemical reduction.   On distillation a fraction was collected which boiled at 98-102° at 13 mm.   The substance had the following composition.

3.620 mg. substance: 7.670 mg. CO_2 and 3.795 mg. H_2O.

$C_5H_{12}O_2$ (104.1).   Calculated.   C 57.65, H 11.62
Found.   " 57.77, " 11.73

The glycol without a solvent possessed a rotation of $\alpha_D^{20} =$ +0.95°.   In absolute alcohol the specific rotation was

$$[\alpha]_D^{20} = \frac{+0.34° \times 100}{1 \times 9.074} = +3.75°$$

Diphenylurethane of Pentanediol-(1,2)—This substance was prepared in the usual manner and recrystallized from dilute alcohol. The urethane melted at 97-98° and had the following composition.

5.660 mg. substance: 0.413 cc. N_2 (24°, 764 mm.).

$C_{19}H_{22}O_4N_2$ (342.2).   Calculated.   N 8.19
Found.   " 8.42

The specific rotation of the urethane was as follows:

$$[\alpha]_D^{20} = \frac{+0.47° \times 100}{1 \times 11.00} = +4.27° \text{ (in absolute alcohol)}$$

Levo-Butanediol-(1,3) from 4-Hydroxybutanone-2

To an actively fermenting sugar solution prepared from 600 gm. of bakers' yeast, 200 gm. of sugar, and 3 liters of tap water, a

solution of 60 gm. of redistilled 1-hydroxybutanone-3 (b.p. 88° at 24 mm.) in an equal amount of water was slowly added drop by drop, with simultaneous addition of 4 liters of a 10 per cent sugar solution. The mixture was mechanically stirred during the addition and for a further 3 days at room temperature. It was then filtered through a Buchner funnel which contained a layer of kieselguhr, and the filtrate was concentrated under reduced pressure. The remaining syrup was extracted twice with a mixture of absolute alcohol and dry ether. The solvents were removed, and on distillation a fraction was received which distilled at between 90–120° at 20 mm. It was strongly levorotatory ($\alpha_D^{25} = -13.6°$). On redistillation a fraction was obtained which boiled at 107–110° at 23 mm. The substance had the following composition.

3.669 mg. substance: 7.261 mg. CO_2 and 3.650 mg. H_2O.
$C_4H_{10}O_2$ (90.1). Calculated. C 53.30, H 11.19
Found.   " 53.96, " 11.13

The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-0.75° \times 100}{1 \times 4} = -18.8° \text{ (in absolute alcohol)}$$

The yield of the levo-1,3-butanediol was not satisfactory. The first fraction obtained on distillation (small amount) was a lower boiling, neutral, levorotatory substance, which was not further examined.

Diphenylurethane of Levo-Butanediol-(1,3)—This urethane was prepared as previously described.[2] It melted at 127–128°. It had the following composition.

8.015 mg. substance: 0.606 cc. N (29°, 758 mm.).
$C_{18}H_{20}O_4N_2$ (328.2). Calculated. N 8.54
Found.   " 8.52

The specific rotation of the urethane in absolute alcohol was as follows:

$$[\alpha]_D^{25} = \frac{-1.85° \times 100}{1 \times 3.59} = -51.5°$$

ON THE CONFIGURATIONAL RELATIONSHIP OF THE CARBINOLS OF THE ISOBUTYL SERIES AND OF ETHYLBENZYLCARBINOL TO THE SIMPLE ALIPHATIC CARBINOLS

BY P. A. LEVENE AND A. WALTI

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On the basis of indirect evidence, Levene and Marker[1] came to the conclusion that configurationally related carbinols of the isopropyl series and of the isobutyl series rotate in opposite directions, the isobutylcarbinols rotating in the same direction as the configurationally related carbinols of the normal series. In the present communication direct chemical evidence is furnished, substantiating the latter conclusion.

The evidence is based on the previously established fact that optically active ethylenic oxides condense with Grignard reagents to form optically active secondary carbinols without undergoing a Walden inversion.[2]

$$CH_2 \rceil$$
$$\qquad O \quad + n\text{-}C_3H_7MgBr \qquad CH_2\text{—}C_3H_7$$
$$HC \lfloor$$
$$\mid \qquad\qquad\qquad\qquad\qquad\qquad HCOH$$
$$CH_3 \qquad\qquad\qquad\qquad\qquad\qquad \mid$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad CH_3$$
$$\text{Dextro} \qquad\qquad\qquad\qquad\qquad \text{Levo}$$
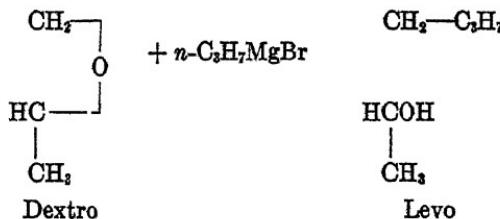
It has now been shown that propylene oxide on condensation with isopropylmagnesium bromide forms methylisobutylcarbinol having the same rotation as the methyl-*n*-butylcarbinol derived from the same oxide; these two carbinols are therefore configurationally related on the basis of the mode of their preparation.

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[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **90,** 669 (1931); **91,** 405 (1931).

[2] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **90,** 81 (1931).

$$CH_3 \quad \mid \quad O + i\text{-}C_3H_7MgBr$$

$$HC \quad \quad \mid \quad CH_3$$

Dextro

$$CH_2 \text{---} CH \overset{CH_3}{\diagdown} CH_3$$

$$HCOH \quad \mid \quad CH_3$$

Levo
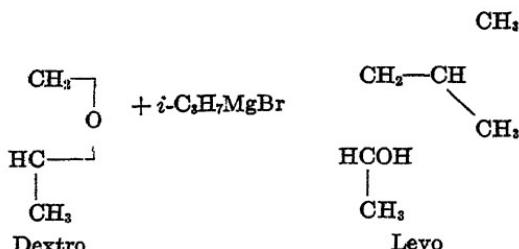
The configurational relationship of ethylisobutyl- and of ethyl-butylcarbinols can be established by a similar procedure; namely,

$$CH_2C_3H_7$$
$$\mid$$
$$HCOH$$
$$\mid$$
$$C_2H_5$$
Levo

$n\text{-}C_3H_7MgBr$

$$CH_2 \quad \mid \quad O$$
$$HC \text{---} \quad \mid \quad C_2H_5$$
Dextro

$i\text{-}C_3H_7MgBr$

$$CH_2CH \overset{CH_3}{\diagup} \diagdown CH_3$$
$$HCOH$$
$$\mid$$
$$C_2H_5$$
Levo
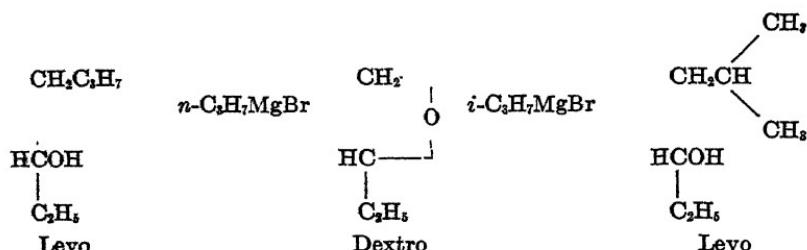
Thus in the case of methyl- and ethylisobutylcarbinols the conclusions regarding the configurational relationships arrived at by the direct method are identical with those reached by the indirect method.

Ethylbenzylcarbinol

The discussion of this section belongs to that of the series of publications on phenylated carbinols. Levene and Stevens[3] on the basis of indirect evidence came to the conclusion that ethyl-benzylcarbinol is configurationally related to ethyl-n-heptylcarbinol rotating in the same direction. This conclusion is now substantiated by direct chemical methods for the reason that the same butylene oxide which leads to levo-ethylbutyl- and levo-isobutylcarbinols leads also to levo-butylbenzylcarbinols.

$$CH_2C_3H_7$$
$$\mid$$
$$HCOH$$
$$\mid$$
$$C_2H_5$$
Levo

$n\text{-}C_3H_7MgBr$ $\longleftarrow$

$$CH_2 \quad \mid \quad O$$
$$HC \text{---} \quad \mid \quad C_2H_5$$
Dextro

$\longrightarrow$ C_6H_5MgBr

$$CH_2C_6H_5$$
$$\mid$$
$$HCOH$$
$$\mid$$
$$C_2H_5$$
Levo
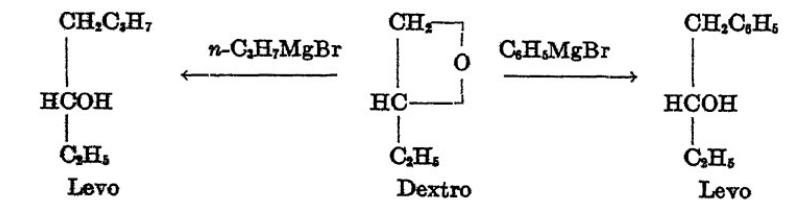
[3] Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, **87**, 375 (1930).

Thus, as a general rule, it seems warranted to assume that phenylated carbinols in which the phenyl group is not directly located on the asymmetric carbon atom rotate in the same direction as the corresponding carbinols of the normal aliphatic series.

<div align="center">EXPERIMENTAL</div>

Levo-Methylisobutylcarbinol from Dextro-Propylene Oxide—0.6 mol of dextro-propylene oxide ($\alpha_D^{24} = +9.2°$) dissolved in 100 cc. of dry ether was added drop by drop to a cooled solution of 0.6 mol of isopropylmagnesium bromide in 250 cc. of dry ether, the mixture being stirred. The mixture was allowed to stand for 7 days at room temperature after which the ether was distilled off. To the residue were added 70 cc. of dry pyridine and 40 gm. of phthalic anhydride. The purified phthalate was steam-distilled in the presence of sodium hydroxide. The distillate was extracted with ether, the ethereal solution being dried and fractionated. The methylisobutylcarbinol distilled at 65–66° at 60 mm. It had the following composition.

3.010 mg. substance: 7.765 mg. CO_2 and 3.675 mg. H_2O.
$C_6H_{14}O$ (102.1).   Calculated.   C 70.52, H 13.81
                        Found.   " 70.35, " 13.66

The rotation of the substance was $\alpha_D^{22} = -4.15°$ (without solvent).

α-Naphthylurethane of Methylisobutylcarbinol—The urethane was prepared in the usual manner. After several recrystallizations from dilute alcohol a substance was obtained which melted at 86–89°. Its analysis was as follows:

9.160 mg. substance: 0.421 cc. N (23.5°, 759 mm.).
$C_{17}H_{21}O_2N$ (271.2).   Calculated.   N 5.17
                              Found.   " 5.28

The specific rotation was

$$[\alpha]_D^{22} = \frac{-0.14° \times 100}{1 \times 3.76} = -3.72° \text{ (in absolute ethyl alcohol)}$$

Dextro-α-Butylene Oxide—This oxide was prepared from the corresponding levo-1-bromo-2-hydroxybutane[4] ($\alpha_D^{24} = -8.2°$) in

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[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **74**, 343 (1927).

a manner similar to that previously described for dextro-propylene oxide.[5] It was observed that although the rotation of this bromohydrin varied with several preparations from $\alpha_D^{26} = -10°$ to $0°$, the butylene oxide derived therefrom was always strongly dextrorotatory ($\alpha_D^{25} = +6.5°$ to $+8.5°$ without solvent). 31 gm. of levo-1-bromo-2-hydroxybutane were dropped into a hot solution (90°) of 12 gm. of sodium hydroxide and 15 cc. of water. The moist dextro-α-butylene oxide distilled off mostly at 61°, the distilling flask being continuously shaken. The distillate weighed 12 gm. and was dried over anhydrous potassium carbonate. The rotation of the α-butylene oxide was $\alpha_D^{26} = +8.75°$ (without solvent).

Levo-Butylene Bromohydrin from Dextro-α-Butylene Oxide—3.6 gm. of dextro-butylene oxide ($\alpha_D^{24} = +8.05°$) were dropped slowly into a cooled solution of 6 cc. of hydrobromic acid (48 per cent) in 6 cc. of water and the mixture was stirred for 30 minutes. Two layers had now formed, the lower layer being separated and dissolved in ether. The aqueous upper layer was extracted with ether and the united ether solutions were washed and then dried over sodium sulfate. On evaporation of the solvent and distillation of the residue, a fraction (5 gm.) was received which distilled at 70–71° at 23 mm. The substance had the composition of butylene bromohydrin.

0.1274 gm. substance: 0.1592 gm. AgBr (Carius).
C_4H_9OBr (153.0).   Calculated.   Br 52.24
                                    Found.   " 53.17

The rotation of the butylene-α-bromohydrin was $\alpha_D^{25} = -6.0°$ (without solvent).

Levo-Ethylisobutylcarbinol from Dextro-α-Butylene Oxide—A solution of 21 gm. of dextro-butylene oxide ($\alpha_D^{24} = +8.05°$) in 50 cc. of anhydrous ether was added drop by drop to an ether solution of 0.6 mol of isopropylmagnesium bromide. The mixture was allowed to stand for 6 days, after which it was worked up in the usual manner. The ethylisobutylcarbinol was purified through the phthalate, and after hydrolysis, isolation, and distillation a fraction was obtained which distilled at 63–64° at 19 mm. The substance had the following composition.

[5] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **68**, 415 (1926).

3.780 mg. substance: 10.020 mg. CO_2 and 4.630 mg. H_2O.
$C_7H_{16}O$ (116.1).   Calculated.   C 72.35, H 13.89
Found.   " 72.30, " 13.70

The rotation of ethylisobutylcarbinol was $\alpha_D^{23} = -3.88°$ (without solvent).

α-Naphthylurethane of Ethylisobutylcarbinol—This urethane was prepared in the usual manner and twice recrystallized from dilute alcohol. It melted at 77–79°.

The analysis of the urethane was as follows:

9.670 mg. substance: 0.443 cc. N (22°, 752 mm.).
$C_{18}H_{23}O_2N$ (285.2).   Calculated.   N 4.91
Found.   " 5.25

The specific rotation of the urethane was as follows:

$$[\alpha]_D^{24} = \frac{-0.15° \times 100}{1 \times 10.00} = -1.50° \text{ (in absolute ethyl alcohol)}$$

Levo-Ethylbutylcarbinol from Dextro-α-Butylene Oxide—A solution of 0.3 mol of dextro-α-butylene oxide ($\alpha_D^{26} = +8.75°$) in 30 cc. of ether was added drop by drop to a stirred solution of 0.3 mol of propylmagnesium bromide in 200 cc. of dry ether. The mixture was allowed to stand for 10 days, after which it was worked up in the usual way. The crude carbinol was slightly dextrorotatory. It was purified through the phthalate. The ethylisobutylcarbinol was now distilled at 66° at 18 mm. The substance had the following composition.

3.640 mg. substance: 9.685 mg. CO_2 and 4.570 mg. H_2O.
$C_7H_{16}O$ (116.1).   Calculated.   C 72.35, H 13.89
Found.   " 72.55, " 14.04

The substance had a rotation of $\alpha_D^{23} = -0.90°$ (without solvent).

α-Naphthylurethane of Ethylbutylcarbinol—This urethane was prepared in the usual manner. It was twice recrystallized from dilute alcohol. It melted at 74–75.5°. The analysis of the substance was as follows:

7.545 mg. substance: 0.340 cc. N (24.5°, 764 mm.).
$C_{18}H_{23}O_2N$ (285.2).   Calculated.   N 4.91
Found.   " 5.19

A 7.5 per cent solution in alcohol showed no rotation in a 1 dm. tube.[6]

Levo-Ethylbenzylcarbinol from Dextro-α-Butylene Oxide—A solution of 0.3 mol of dextro-α-butylene oxide in 70 cc. of dry ether was added drop by drop to a stirred solution of 0.3 mol of phenyl-magnesium bromide in 200 cc. of ether. After standing for 4 days the mixture was worked up in the usual way. The crude carbinol was purified through the phthalate. The ethylbenzylcarbinol distilled at 126° at 27 mm. It had the following composition.

3.090 mg. substance: 9.065 mg. CO_2 and 2.605 mg. H_2O.
$C_{10}H_{14}O$ (150.1). Calculated. C 79.95, H 9.40
Found. " 80.00, " 9.43

The substance had a rotation of $\alpha_D^{24} = -12.2°$ (without solvent).

α-Naphthylurethane of Levo-Ethylbenzylcarbinol—This urethane was prepared in the usual way. It was recrystallized from dilute alcohol and melted at 116–119°. It had the following composition.

6.670 mg. substance: 0.281 cc. N (27°, 752 mm.).
$C_{21}H_{21}O_2N$ (319.2). Calculated. N 4.39
Found. " 4.74

The specific rotation of the substance in absolute alcohol was approximately $\alpha_D^{25} = -16°$.

[6] Owing to the low rotation of the carbinol, the racemization of the urethane was complete.

ULTRAFILTRATION

I. ULTRAFILTRATION OF ELECTROLYTES FROM ALKALI CASEINATE SOLUTIONS*

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Ultrafiltration is an important research tool in colloid chemical and in certain types of biological investigation. However, in spite of the many years that ultrafiltration has been used as a research tool, the phase of the subject concerning the ultrafiltration of electrolytes from systems containing electrically charged colloids has been lacking in both experimental data and a theoretical basis for the interpretation of the process. The widely current view is that an ultrafiltration of this kind is a simple filtration and the diffusible electrolytes ultrafilter through in the same concentration that they are present in the free solvent of the colloidal solution. Differences that are found by gross analysis are ascribed to such effects as solvation of the colloid or to adsorption. On the basis of such an assumption for ultrafiltration, the amount of peptizing electrolyte adsorbed by a colloid has been evaluated by subtracting the concentration in the ultrafiltrate from the total of the peptizing electrolyte in the colloidal solution. In a similar way what has been taken to be the hydration of certain colloids has been calculated from the increase of the concentration in the filtered liquid of an added electrolyte reference substance. In this way in recent years ultrafiltration has been employed to evaluate the constitution of stannic oxide sols peptized by potassium hydroxide, chromic oxide sols peptized by ammonium chlo-

ride, and ferric oxide sols, by Wintgen and his coworkers (1–3). Determination of the supposed hydration of soaps by ultrafiltration has been carried out by McBain and Jenkins (4) on potassium laurate and sodium oleate using the corresponding alkali chlorides as reference substances.

In this communication, as the first results of a study of this subject, data on alkali caseinate systems will be presented which show that ultrafiltration of a diffusible electrolyte from systems containing charged colloids is not a simple filtration in which the electrolyte comes through in the ultrafiltrate liquid in unchanged concentration. Instead, there is produced a very definite difference in concentration between the electrolyte in the ultrafiltrate as against its concentration in the colloidal solution aside from any effects due to adsorption or hydration. The differences in concentration between the ionic constituents of the ultrafiltrate and their concentration in the colloidal solution, even though ultrafiltration is a kinetic, irreversible process, are found to partake of the nature of a Donnan (5) membrane distribution. The distribution found in this type of ultrafiltration seems to follow as a consequence of the extension of the Donnan theory to heterogeneous equilibria by Wilson and Wilson (6), Oakley (7), and other workers. A quotation from Oakley illustrates the reasoning involved.

"The important condition for the establishment of a Donnan equilibrium is that one kind of an ion shall be prevented from diffusing where all the others can so diffuse. This condition is fulfilled in the case of surface ionization. Here the diffusion of one kind of an ion is prevented, not by a membrane, but by its forming a structural part of a large molecular aggregate. The idea of a membrane, is therefore to be replaced by the idea of a certain boundary near the surface of the particle beyond which the anions or cations, which are 'partners' to the colloidal cations or anions as the case may be, are unable to diffuse because of electrostatic attraction."

The application of the extension of the Donnan theory to ultrafiltration was advanced by Greenberg and Gunther ((8) p. 501) in a paper on the diffusible calcium of blood serum, where it is stated: ". . . it is postulated that there is the same spatial arrangement of ionic constituents in the vicinity of a colloidal micelle as there is obtained with a membrane interposed. On this view, ultrafiltration would give in essence the same experimental

results as a membrane distribution experiment." In the same paper the results of the ultrafiltration of chloride ion from blood serum are given as evidence favoring the hypothesis advanced for this type of ultrafiltration. Admittedly, because of the small Donnan effect obtainable with blood serum, the chloride results are not an exacting test.

In the literature, mention of a Donnan effect in ultrafiltration has also been made by Augsberger (9) and by Duclaux and Titeica (10) but no mathematical or experimental data are offered in either publication.

EXPERIMENTAL

In the experimental work, alkali caseinate solutions were prepared from pure isoelectric casein (11) by adding an amount of either sodium or potassium hydroxide sufficient to give a reaction between pH 6.5 to about 8.0. Collodion membranes for the filtration were prepared from either parlodion or Scherings brand, dissolved in a mixture of alcohol and ether and containing 5 cc. of ethylene glycol to each 100 cc. The membranes were cast on the inside of 25 cc. conically pointed centrifuge tubes. The rest of the ultrafiltration procedure and the apparatus used are the same as has been described by Greenberg and Gunther (8) for the determination of diffusible calcium. Since no protein came through into the ultrafiltrate, as was shown by test, the ultrafiltrates obtained consisted simply of aqueous solutions of the salt added to the respective caseinate solutions. To determine the salt concentrations in the ultrafiltrate samples, the specific conductance at 25° was measured, and the concentration corresponding to the conductance was read off from curves prepared from calibrating solutions. All solutions were prepared and analyzed in terms of gm. equivalents per liter of water at 20°.

To carry out an experiment, a volume of caseinate solution sufficient completely to fill the membrane, between 20 and 24 cc., was introduced into the bag, and the first few drops that appeared on the outer surface of the membrane after filtration commenced were wiped off to avoid the water contained in the pores of the membrane. The filtration was then allowed to proceed until 3 cc. were obtained for a sample, which was then removed and analyzed for salt concentration. As many 3 cc. samples as it was possible to ultrafilter through were obtained in each experiment.

The data are given in Tables I to V. Tables I and II are the results on sodium caseinate to which sodium chloride and sodium sulfate respectively have been added and Tables III to V give the results with potassium caseinate solutions containing potassium chloride, potassium acetate, and potassium oxalate in the stated order. Table III for potassium caseinate with potassium chloride contains the results of two series at different ratios of potassium hydroxide to casein. As Table III shows, changing the ratio of

TABLE I

Sodium Caseinate + NaCl

Casein	NaOH added	NaCl added	NaCl in filtrate	Calculated NaCl in filtrate	$\dfrac{F_{NaCl}}{C_{NaCl}}$	$\dfrac{F_{NaCl}}{O_{NaCl}}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
per cent	N	N	N	N		
1.95	0.0131	0.0062	0.0104	0.0109	0.95	1.68
1.95	0.0131	0.010	0.0149	0.0151	0.99	1.53
1.30	0.0088	0.0114	0.0152	0.0151	1.00	1.34
1.75	0.0115	0.0148	0.0191	0.0197	0.97	1.32
1.75	0.0115	0.0246	0.0302	0.0300	1.00	1.23
2.82	0.0197	0.0443	0.0520	0.0532	0.98	1.18

TABLE II

Sodium Caseinate + Na$_2$SO$_4$

Casein	NaOH added	Na$_2$SO$_4$ added	Na$_2$SO$_4$ in filtrate	Calculated Na$_2$SO$_4$ in filtrate	$\dfrac{F_{Na_2SO_4}}{C_{Na_2SO_4}}$	$\dfrac{F_{Na_2SO_4}}{O_{Na_2SO_4}}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
per cent	N	N	N	N		
2.78	0.0196	0.0121	0.0207	0.0230	0.90	1.71
1.68	0.0115	0.0186	0.0250	0.0256	0.98	1.34
1.97	0.0131	0.0242	0.0304	0.0323	0.94	1.24

alkali to casein over this restricted range produces no experimental alterations. The tables give the casein content, the amount of alkali hydroxide and salt added to the casein in terms of equivalents per liter of water, the analytically determined concentration of salt in the filtrate, and the calculated salt concentration. Finally, in the last two columns are given the ratio of the salt in the filtrate to the calculated salt, designated by $\dfrac{F_{salt}}{C_{salt}}$ and the ratio

TABLE III

Potassium Caseinate + KCl

Casein	KOH added	KCl added	KCl in filtrate	Calculated KCl in filtrate	$\dfrac{F_{KCl}}{C_{KCl}}$	$\dfrac{F_{KCl}}{O_{KCl}}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
per cent	N	N	N	N		
2.80	0.0177	0.005	0.009	0.0106	0.85	1.80
2.23	0.0141	0.006	0.010	0.0110	0.91	1.75
0.64	0.0106	0.004	0.0069	0.0076	0.91	1.73
1.38	0.0088	0.005	0.0077	0.0083	0.93	1.56
2.41	0.0211	0.0202	0.0265	0.0289	0.92	1.39
0.74	0.0044	0.0075	0.0097	0.0094	1.03	1.30
1.63	0.0101	0.0152	0.0188	0.0196	0.96	1.24
1.28	0.0053	0.007	0.0084	0.0093	0.90	1.20
2.25	0.0123	0.0329	0.0392	0.0386	1.02	1.20

0.5 to 0.6 mm KOH per gm. casein

0.8 to 0.9 mm KOH per gm. casein

1.75	0.0141	0.004	0.0074	0.0085	0.87	1.85
1.46	0.0117	0.005	0.0084	0.0092	0.91	1.68
1.25	0.0101	0.006	0.0090	0.0098	0.92	1.50
3.33	0.0302	0.0202	0.0288	0.0319	0.90	1.43
0.94	0.0076	0.007	0.0094	0.0101	0.93	1.33
2.22	0.0201	0.0303	0.0352	0.0391	0.90	1.16

TABLE IV

Potassium Caseinate + Potassium Acetate

Casein	KOH added	$KC_2H_3O_2$ added	Urea added	$KC_2H_3O_2$ in filtrate	Urea in filtrate	Calculated $KC_2H_3O_2$ in filtrate	$\dfrac{F_{KC_2H_3O_2}}{C_{KC_2H_3O_2}}$	$\dfrac{F_{KC_2H_3O_2}}{O_{KC_2H_3O_2}}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
per cent	N	N	M	N	M	N		
1.00	0.0096	0.0050		0.0078		0.0085	0.92	1.56
2.04	0.0192	0.0299		0.0357		0.0383	0.93	1.19
1.75	0.0101	0.0199	0.0130	0.0223	0.0132	0.0245	0.91	1.12
1.18	0.0067	0.0299	0.0130	0.0312	0.0130	0.0383	0.94	1.04

of the analytically determined salt concentration in the filtrate
to the original amount of salt in the caseinate solution, this being
designated by $\dfrac{F_{salt}}{O_{salt}}$.   In Tables IV and V, on the acetate series

and the oxalate series, are also given the results of a few preliminary tests in which a small amount of urea was added to the caseinate solution.

The calculations for the concentrations of salt theoretically expected were based on the following treatment. If the hypothesis that there is a Donnan membrane distribution between the electrically charged colloidal micelle and the intermicellar liquid is accepted, then the ultrafiltrate at any moment gives a picture of the composition of the intermicellar liquid, since the colloidal micelle is held back by the membrane. If now from a large volume of a given colloidal solution, e.g. sodium caseinate containing a salt of the type of sodium chloride, there is ultrafiltered a small amount of liquid, so no appreciable change results in the compo-

TABLE V

Potassium Caseinate + Potassium Oxalate

Casein	KOH added	$K_2C_2O_4$ added	Urea added	$K_2C_2O_4$ in filtrate	Urea in filtrate	Calculated $K_2C_2O_4$ in filtrate	$\dfrac{F_{K_2C_2O_4}}{C_{K_2C_2O_4}}$	$\dfrac{F_{K_2C_2O_4}}{O_{K_2C_2O_4}}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
per cent	N	N	M	N	M	N		
3.46	0.0289	0.0185	0.0102	0.0239	0.010	0.0346	0.69	1.29
1.44	0.0126	0.0146	0.0095	0.0167	0.0099	0.0221	0.76	1.14
2.30	0.0193	0.0278		0.0310		0.0395	0.79	1.11

sition of the colloidal solution, the concentration of sodium chloride in the ultrafiltrate is given by:

$$X = \sqrt{Y_0 \, (Y_0 + Z_0)} \tag{1}$$

where X is the sodium chloride concentration of the ultrafiltrate, Y_0 is the concentration of sodium chloride in the caseinate solution, and Z_0 is the sodium ion combined with the casein.

This equation further assumes that the concentrations do not differ from the activities of the corresponding ions. Similarly, when the solution is sodium caseinate, containing a salt of the valence type of sodium sulfate, the sodium sulfate of the ultrafiltrate is given by the equation

$$\sqrt[3]{Y_0 \, (Y_0 + Z_0)^2} \tag{2}$$

in which X now represents the equivalent concentration of sodium sulfate in the ultrafiltrate, Y_0 the sodium sulfate of the colloidal solution, and Z_0 the sodium equivalence of the casein.[1] It is pointed out[1] that in actual practice the simple equations, Equations 1 and 2, are theoretically not valid for calculating the expected ultrafiltration values, and a decrease in the ultrafiltrate concentration is to be expected as the volume filtered increases. Actually, experimentally it was found that instead of obtaining a decreasing concentration of salt with increase in the fraction ultrafiltered, the concentration in the ultrafiltrate remained constant and un-

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[1] When the amount of ultrafiltrate removed is considerable, so that significant changes are produced in the composition of the colloidal solution, Equations 1 and 2 should no longer hold and an equation in terms of the fraction of the volume ultrafiltered and the rate of change of the ions of the colloidal solution would be expected to apply. Such an equation can be developed. The change of the individual ionic components with change in volume due to ultrafiltration in a system of the type of sodium caseinate solution containing sodium chloride is given by:

$$Z = Z_0 \frac{V_0}{V_0 - V}; \; Y = \frac{Y_0 V_0 - X V}{V_0 - V}; \; X = \frac{Y_0 V_0 - Y \, (V_0 - V)}{V}$$

in which Z, Y, and X represent their respective ionic components at any given stage in the ultrafiltration, Z_0 and Y_0 represent the original concentrations in the colloidal solution, V_0 is the original volume, and V is the volume ultrafiltered. Substituting the values of Y and Z in the Donnan equation, $X^2 = Y \, (Y + Z)$ there is obtained

$$X^2 \, (V_0 - V)^2 = (Y_0 V_0 - X V)^2 + Z_0 V_0 \, (Y_0 V_0 - X V)$$

From this, carrying through the necessary simplification and substituting the fraction of the volume ultrafiltered $\dfrac{V}{V_0} = N$ there is obtained:

$$X^2 \, (1 - 2N) + X N \, (2Y_0 + Z_0) = (Y_0^2 + Y_0 Z_0)$$

and

$$X = \frac{\sqrt{[N \, (2Y_0 + Z_0)]^2 + 4 \, (Y_0^2 + Y_0 Z_0) \, (1 - 2N)} - N \, (2Y_0 + Z_0)}{2(1 - 2N)} \tag{3}$$

This equation becomes very much simplified when half of the solution is ultrafiltered; i.e., when $N = \frac{1}{2}$. At this point $1 - 2N$ becomes zero and the equation reduces to

$$X = 2 \frac{Y_0^2 + Y_0 Z_0}{2Y_0 + Z_0} \tag{4}$$

On applying Equation 3, it is found that the concentration of the ultrafiltrate salt, X, should decrease as N increases, the magnitude of the decrease being determined by the ratio of Y_0 to Z_0. When Y_0 is small in comparison with Z_0 the expected falling off is considerable, when the reverse is true the decrease should become negligible.

changed, to within about 1 per cent in all samples, for as large a fraction of the solution as it was possible to ultrafilter. The salt concentration in the last 3 cc. sample differed only in an insignificant degree from that in the first 3 cc. sample in any one experiment. Accordingly, the figures given in all the tables are the average values for all the samples of each experiment and the expected values have been calculated according to Equations 1 and 2 instead of Equation 3.[1] Further consideration of this point will be reserved until other colloidal systems have been examined.

The data strongly favor the view that this type of ultrafiltration partakes of the nature of a Donnan membrane distribution. The results show that the increase in concentration of the diffusible salt in the ultrafiltrate over its concentration in the original caseinate solution cannot be due to a removal of free solvent water by hydration of the casein. If hydration were the responsible factor one would expect a concentration increase proportional to the amount of casein in the solution and this is not found. Instead, the ratio of the salt in the ultrafiltrate to the salt in the original caseinate solution (figures for which are given in the last column of the tables) depends, as would be expected, on the membrane distribution hypothesis, upon the amount of diffusible salt present in comparison with the concentration of the alkali cation paired with the casein in the solution. The lower the concentration the added salt is, in comparison to the equivalent concentration of the casein, the larger should be the ratio of the ultrafiltrate salt to the original salt concentration of caseinate solution, and the greater the concentration of the added salt, the more closely should the ultrafiltrate salt approach the salt concentration of the caseinate solution. That this is what actually takes place is borne out by the tabulated data. To the evidence given may be added the results of the few preliminary ultrafiltration experiments with the acetates and oxalates which contained a small amount of urea. The results, as recorded in Tables IV and V, show the urea came through in the ultrafiltrate in the same concentration it was present in the original solution. Since urea, being a non-electrolyte, is exempt from the Donnan effect, the findings reduce the possibility of hydration to a small factor.

Qualitatively, from what has been stated above, the experimental results are in good agreement with the membrane distri-

bution theory. Quantitatively, it has already been noted that the deductions from Equation 3 are not borne out by the caseinate systems. On the other hand, the agreement with the basic equations for the Donnan equilibrium is reasonably good. In Column 5 of Tables I to III and in Column 7 of Tables IV and V, are given the calculations obtained according to Equations 1 and 2 for the calculated salt in the filtrate. In Column 6 of Tables I to III and Column 8 of Tables IV and V are given the ratios of the analytically determined salt to the calculated salt. It is noticeable that the calculated values for the salt concentration of the ultrafiltrate tend to be somewhat higher than the determined values. In Column 6 of Tables I to III and Column 8 of Tables IV and V, giving the ratios between the determined and calculated values, the ratios for the monovalent salts are seen to average about 0.95. The same is true for the sodium sulfate series. The oxalate series give still lower ratios of about 0.75. This effect follows from using the concentration values instead of the activities of the ions. It is reasonable to suppose that in the presence of the protein the activity coefficients of the respective ions are lower than in the aqueous filtrate solution. Since the magnitude of the effect produced by the casein is as yet unknown, it is not at present possible to apply a correction. The especially great lowering of the oxalate is probably to be ascribed to the formation of complexes with the casein, still further reducing the activity of the oxalate ion. Evidence for such complex formation has previously been brought forward by one of the writers (12) by a study of the transference numbers of protein solutions.

The type of ultrafiltration discussed in this paper has a number of significant applications both in pure colloid chemistry and in biology. It is obvious that the effect noted here must be taken into account where ultrafiltration is employed in evaluating the constitution of colloids. Furthermore, it seems feasible to employ this type of ultrafiltration as a means of studying the effect of colloidal systems on the activity coefficients of ions.

Biologically, the phenomenon provides a basis for the understanding of the nature of the distribution of the calcium of the blood serum into diffusible and non-diffusible calcium, which up to now has had only an empirical significance. The results of the blood studies by ultrafiltration are now seen to be no different in principle from the results by compensation dialysis.

The other important biological application is in connection with the excretion of the urine by the kidney. Current theories of kidney function (13) hold that glomerular urine formation is purely an ultrafiltration of the colloid-free plasma constituents. To establish this theory experimentally, investigators, most recently Freeman, Livingston, and Richards (14), have attempted to demonstrate the equality of chloride ion concentration in plasma and glomerular urine. From the treatment outlined here, it is to be seen that no such equality should occur. Instead, the chloride content of glomerular urine should be at least 10 per cent greater than the plasma chloride concentration (8). Similar effects should also be found for the other ionic constituents of glomerular urine.

SUMMARY

1. Experimental evidence is brought forward to support the hypothesis that the ultrafiltration of diffusible electrolytes from systems containing electrically charged colloids partakes of the nature of a Donnan membrane distribution.

2. The applications of the phenomenon of this type of ultrafiltration to certain colloidal and biological problems are pointed out.

BIBLIOGRAPHY

1. Wintgen, R., *Z. physik. Chem.*, **103**, 238 (1923).
2. Wintgen, R., and Biltz, M., *Z. physik. Chem.*, **107**, 403 (1923).
3. Wintgen, R., and Löwenthal, H., *Z. physik. Chem.*, **109**, 378 (1924).
4. McBain, J. W., and Jenkins, W. J., *J. Chem. Soc.*, **121**, 2325 (1922).
5. Donnan, F. G., *Chem. Rev.*, **1**, 73 (1924).
6. Wilson, J. A., and Wilson, W. H., *J. Am. Chem. Soc.*, **40**, 886 (1918).
7. Oakley, H. H., *J. Physic. Chem.*, **30**, 902 (1926).
8. Greenberg, D. M., and Gunther, L., *J. Biol. Chem.*, **85**, 491 (1929–30).
9. Augsberger, A., *Ergebn. Physiol.*, **24**, 618 (1925).
10. Duclaux, J., and Titeica, R., *Compt. rend. Acad.*, **189**, 101 (1929).
11. Greenberg, D. M., and Schmidt, C. L. A., *J. Gen. Physiol.*, **7**, 287 (1924).
12. Greenberg, D. M., *J. Biol. Chem.*, **78**, 265 (1928).
13. Cushny, A. R., The secretion of the urine, London, 2nd edition (1926).
14. Freeman, B., Livingston, A. E., and Richards, A. N., *J. Biol. Chem.*, **87**, 467 (1930).

MINERAL METABOLISM DURING INVOLUTION OF SIMPLE GOITER*

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METZGER

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The fact that rabbits, fed on an exclusive diet of cabbage, or other member of the brassica family, will develop simple goiter, furnishes an excellent method for the study of this phase of thyroid physiology. By this means thyroid insufficiency (hyperplasia) may be produced which can, by the administration of a few mg. of iodine, be restored in a short time to the normally functioning (colloid-containing) gland. Further, if, after a thyroid of several gm. has been produced by cabbage feeding, daily iodine administration is continued, a state of temporary overfunctioning results (Webster and Chesney (1)). It appears that the goiterous rabbit has an exaggerated reaction to iodine. Man seems to have more control over the excretion of thyroid hormone under such conditions while in the rabbit an excess of thyroid secretion occurs as is indicated by the abnormally high level of metabolism and loss of weight, followed in some instances by death. This, however, requires further study.

In this paper we are reporting results of a study of the mineral metabolism of three rabbits in varying states of thyroid activity. Simple goiters were produced by feeding cabbage until the thyroids became palpable. The rabbits were then placed in metabolism cages and the food intake accurately measured and analyzed. The excreta were collected and analyzed for two 3 day periods.

A few mg. of potassium iodide were then injected intraperitone-
ally and the metabolism study continued for three 3 day periods
and two 6 day periods during which time the cabbage diet was
continued. At the end of each period the bladders were emptied
by compressing the lower abdomen. We have found that rab-
bits remain in excellent nutritive conditions for several months

TABLE I—*Mineral Metab*

The results are expressed in gm.

Period No.*	Weight	Sodium			Potassium			Calcium			Ma	
		Intake	Out-put†	Balance	Intake	Out-put†	Balance	Intake	Output	Balance	In-take	C
											Rabbi	
I	2400	0.255	0.180	+0.075	3.44	3.10	+0.34	1.005	0.551	+0.454	0.270	
II	2490	0.252	0.140	+0.112	3.39	2.71	+0.68	0.992	0.465	+0.527	0.267	
III	2530	0.177	0.080	+0 097	3.78	3.01	+0.77	0.908	0.414	+0.494	0.250	
IV	2590	0.173	0.034	+0.139	3.66	2.88	+0.78	0.878	0.423	+0.455	0.24	
V	2600	0.153	0.115	+0.038	3.24	2.49	+0.75	0.777	0.620	+0.157	0.21	
VI	2580	0.398	0.210	+0.188	7.31	6.34	+0.97	1.65	1.070	+0.580	0.484	
VII	2600	0 406	0.310	+0 096	7 46	5.40	+2.06	1.68	0.887	+0.793	0.494	
											Rab	
I	1740	0.204	0.107	+0.097	2.75	2.29	+0.46	0.804	0.559	+0.245	0.216	
II	1840	0.168	0.027	+0.141	2.27	1.95	+0.32	0.636	0.328	+0.308	0.178	
III	1730	0.122	0.071	+0.051	2.56	2.17	+0.39	0.619	0.618	+0.001	0.172	
IV	1710	0.144	0.227	−0.083	3.05	2.13	+0.92	0.732	0.433	+0.299	0.204	
V	1660	0.139	0.051	+0.088	2.94	2.30	+0.64	0.706	0.331	+0.375	0.197	
VI	1610	0.293	0.190	+0.103	5 38	4.67	+0.71	1.21	0.775	+0.435	0.355	
VII	1660	0.345	0.215	+0.130	6.32	4.40	+1.92	1.43	0.920	+0.510	0.418	

* Periods I to V are 3 days each while Periods VI and VII are 6 days each. At the en
† No determination on feces.

on a diet consisting solely of cabbage, though ordinarily we add
a small amount of alfalfa and oats once or twice weekly.   In the
experiments reported here only cabbage was fed in order to simplify
the analytical problems.

Inorganic constituents of vegetables vary considerably in the
same lot.   For example, it is very difficult to get a fair sample from
a bushel basket of carrots and still more difficult to feed a fair
sample of such carrots *daily* to the animals.   The mineral con-

tent of one bag of oats may show significant variations from that of another bag of the same lot. Because of such experiences we chose heads of cabbage large enough for the daily ration of these rabbits and from each of them we cut a fair segment of 100 gm. For each two periods these were all pooled and dried at 100°, ground, ashed, and analyzed.

ʋolism on Cabbage Diet

gnesium		Chlorine			Phosphorus			Sulfur			Nitrogen		
ut-put	Balance	In-take	Output	Balance	In-take	Out-put	Balance	In-take	Output	Balance	In-take	Out-putt†	Balance
t 641													
165	+0.105	0.558	0.772†	−0.214	0.477	0.132	+0.345	1.18	1.04†	+0.14	3.59	2.44	+1.15
130	+0.137	0.551	0.459†	+0.092	0.471	0.120	+0.351	1.17	0.904†	+0.266	3.54	2.62	+0.92
139	+0.111	0.443	0.559†	−0.116	0.535	0.138	+0.397	1.18	0.963†	+0.217	3.52	2.66	+0.86
127	+0.117	0.419	0.481†	−0.062	0.517	0.197	+0.320	1.14	0.938†	+0.202	3.40	2.86	+0.54
296	−0.080	0.371	0.410	−0.039	0.453	0.442	+0.011	1.008	0.907	+0.101	3.01	2.24	+0.77
257	+0.227	0.737	0.774	−0.037	1.03	0.603	+0.427	1.907	1.902	+0.005	7.08	5.87	+1.21
306	+0.188	0.752	1.008†	−0.256	1.05	0.414	+0.636	1.95	1.74†	+0.210	7.22	5.32	+1.90
t 657													
170	+0.046	0.446	0.576	−0.130	0.382	0.176	+0.206	0.946	0.820	+0.126	2.87	1.90	+0.97
092	+0.086	0.368	0.247†	+0.121	0.315	0.042	+0.273	0.780	0.676†	+0.104	2.37	2.30	+0.07
199	−0.027	0.295	0.394†	−0.099	0.364	0.294	+0.070	0.803	0.710†	+0.093	2.39	2.53	−0.14
169	+0.035	0.349	0.500†	−0.151	0.431	0.113	+0.318	0.950	0.745†	+0.205	2.83	2.20	+0.63
140	+0.057	0.337	0.409†	−0.072	0.415	0.112	+0.303	0.916	0.700†	+0.216	2.73	2.02	+0.71
289	+0.066	0.542	0.632†	−0.090	0.755	0.333	+0.422	1.40	1.35†	+0.050	5.21	5.03	+0.18
292	+0.126	0.638	0.861†	−0.223	0.888	0.377	+0.511	1.65	1.35	+0.300	6.13	5.06	+1.07

of Period II in each case 10 mg. of KI were injected intraperitoneally.

Methods of Analysis—All determinations were made in duplicate. Nitrogen was determined by the Kjeldahl method. Most of the inorganic constituents of urine, feces, and food were determined on the ash obtained by incinerating in platinum dishes in a muffle furnace at 400–450° for about 18 hours. It was usually necessary to extract the ash with hot water containing a little dilute hydrochloric acid. This was filtered and the insoluble residue dried and ashed for another period of 18 hours, reextracted,

combined with the first extract, and made up to a definite volume, usually 100 cc.

Sodium and potassium were determined by the excellent perchlorate method of Smith and Ross (2), after removal of alkali earths, phosphates, and sulfates, etc., with barium hydroxide, ammonium carbonate, and ammonium oxalate. The combined chlorides were weighed, after which the potassium was determined and sodium calculated.

Calcium was determined on the ash extract by the method of Shohl and Pedley (3); magnesium, by McCrudden's method (4); total phosphorus, by precipitation as the phosphomolybdate which was dissolved in standard alkali and excess of alkali titrated (5).

TABLE II

Mineral Metabolism on Diet of Alfalfa Hay and Oats, for Period of 7 Days
The results are expressed in gm.

Rabbit No.	Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance
	Sodium			Potassium			Calcium		
625	0.210	0.262	−0.052	3 67	5.47	−1.80	4.54	2.32	+2.22
635	0.210	0.30	−0.090	3 67	4.99	−1.32	4.54	2.66	+1.88
	Magnesium			Chlorine			Phosphorus		
625	0.717	0.90	−0.183	0.731	0 63	+0.101	1.25	1.48	−0.23
635	0.717	1.06	−0.343	0.731	0.82	−0.089	1.25	1.46	−0.21

Chlorides in urine were estimated by the Volhard-Arnold method, and in food and feces by ashing with sodium carbonate and titrating the extracted ash with silver nitrate.

Sulfur in urine was determined by Benedict's method (6) and in food and feces by fusing with sodium carbonate and sodium peroxide and weighing the sulfur as barium sulfate.

Results—The analytical data of two of the three rabbits studied are given in Table I. The results of the third rabbit are similar and are, therefore, not given. For comparison the mineral balance of two rabbits, Rabbits 625 and 635, on our stock diet of alfalfa and oats was determined over a period of 1 week and the results of this study will be found in Table II. The analyses of the cabbage fed will be found in Table III, and the amounts eaten, in Table IV.

Cabbage is digested nearly completely by the rabbit and the weight of feces is consequently small. The amount obtained was too little to make a complete analysis. Sodium, potassium, chlorine, and sulfur estimations were not made. Since the excretion of these elements in the feces amounts to less than 5 per cent in most cases—occasionally a little more in the case of sulfur—no serious error arises in considering the urinary output the total.

TABLE III

Analyses of Cabbage Fed

Period No.	Sodium	Potassium	Calcium	Magnesium	Chlorine	Phosphorus	Sulfur
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I–II	0.017	0.229	0.067	0.018	0.0372	0.0318	0.0788
III–V	0.012	0.254	0.061	0.017	0.0291	0.0359	0.0792
VI–VII	0.014	0.257	0.058	0.017	0.0259	0.0361	0.0670

TABLE IV

Cabbage Eaten

Period No.	Rabbit 641	Rabbit 657
	gm.	*gm.*
I	1500	1200
II	1481	990
III	1489	1014
IV	1439	1200
V	1274	1157
VI	2846	2092
VII	2903	2462

No significant changes are noted in chlorine, potassium, or total sulfur elimination or balance during varying stages of thyroid activity, but definite alterations in the balance of the alkali earths, phosphorus, and possibly sodium do occur, as the thyroid changes from an underfunctioning hyperplastic gland to a normal, colloid-containing one and as the total metabolism changes from low to normal.

Calcium—Rabbits on a cabbage diet are in positive calcium balance when the thyroid is hyperplastic. The retention amounts

to 0.3 to 0.5 gm. of calcium in a 3 day period.   After the giving of 2 or 10 mg. of potassium iodide intraperitoneally, thus restoring the hyperplastic thyroid to the normal colloid-containing gland (Marine and Williams (7)), Rabbits 657 and 638 are practically in calcium balance while in Rabbit 641 the positive balance is reduced from about 0.5 gm. to 0.16 gm. This change occurs during the first period (1st to 3rd day) after iodine is given in the case of Rabbit 657; in Rabbits 641 and 638, during the 3rd period (7th to 9th day).   It is brought about through an increase in the calcium excretion amounting to a 20 per cent rise in Rabbit 641 and 50 per cent in Rabbits 638 and 657.

On our stock diet of alfalfa hay and oats (Table II, Rabbits 625 and 635), we find a large positive calcium balance also.   This is probably due to the fact that the intake of calcium is 2 to $2\frac{1}{2}$ times as great as on the cabbage diet.   That the calcium retention of the rabbits with goiter is associated with thyroid hyperplasia is evident from the effect of iodine in reducing the positive balance to zero *while still on the cabbage diet*.   After the effect of iodine has worn off and the thyroid begins to undergo hyperplasia again the positive calcium balance becomes as great as before.

Magnesium metabolism parallels that of calcium.   While the rabbits have palpable goiters the balance is positive; on administering iodine it becomes negative in each instance, and this transition from positive to negative magnesium balance occurs in the same periods with the change in calcium; *i.e.*, in the first after period with Rabbit 657 and the 3rd after period with the other two.   Here also the negative balance is brought about by an increase in magnesium excretion amounting to 50 to 100 per cent.

On the alfalfa and oat diet the magnesium intake is slightly greater than on cabbage, but the balance is slightly negative. This contrasts sharply with calcium.   Here there is no excessive intake, as in the case of calcium, to mask the change in balance. When rabbits have simple goiter, they are in positive magnesium balance.   When the gland is involuted with a little iodine, the balance becomes negative.   On continued cabbage feeding the thyroid returns to the hyperplastic state and there is again a retention of magnesium.   If one makes the not unreasonable assumption that in these changes calcium and magnesium follow similar paths, we have additional reason for inferring that cal-

cium retention is directly associated with the development of simple goiter.

Potassium—There is no clear indication that potassium metabolism is affected by changes in thyroid activity. The balance is positive while the rabbits have hyperplastic thyroid and remains so after its involution. It should be noted that the intake of potassium on a cabbage diet is more than twice that on our stock diet of alfalfa and oats. On this lower potassium intake the balance is negative.

Sodium—On a diet of alfalfa and oats rabbits are in slight negative sodium balance. This is probably associated with the low sodium content of these foods. On cabbage the intake of sodium is 2 or 3 times greater than on the stock diet and the rabbits retain sodium. While the thyroids are undergoing involution, however, there is a tendency for the sodium intake and output to be nearly equal, especially at the time when the effects of iodine on calcium and magnesium balances are most marked.

Phosphorus—Variations in phosphorus metabolism follow closely those of magnesium and calcium. When the balances of the latter elements are changed from positive to negative by the action of iodine, the phosphorus excretion rises and results in a very much lower retention than is found in the goiterous rabbits. As the cabbage diet is continued and the thyroid again becomes hyperplastic, the phosphorus excretion diminishes and the original large positive balance is restored. On the stock diet phosphorus intake is much lower and the balance is slightly negative.

Chlorine, Sulfur—No alterations were noted in the metabolism of chlorine and total sulfur.

<div align="center">DISCUSSION</div>

Aub, Bauer, Heath, and Ropes (8) in a series of excellent metabolism experiments demonstrated that in Graves' disease calcium elimination is markedly increased above the normal, resulting in a negative balance. They were able to show this convincingly by the expedient of placing their subjects on a very low calcium intake. Previous studies had been inconclusive and contradictory because unsatisfactory methods were used. The earlier work has been reviewed by these investigators and will not be discussed here. Phosphorus excretion in Graves' disease was also

above the normal. Evidence was adduced to show that the source of the calcium and phosphorus lost in Graves' disease was bone, and they were inclined to attribute this calcium and phosphorus loss to a "stimulating catabolic effect on the calcium deposits in the bones." In myxedema, on the other hand, they were able to show that the calcium elimination was less than normal, although, because of the abnormally low calcium intake the balance here was also negative but only slightly so.

Thyroid medication in these patients restored this low calcium excretion to the normal while in normal persons it increased the calcium elimination.

That the thyroid enlargement caused by feeding cabbage to rabbits is simple goiter is proved by the low metabolism that obtains and by other symptoms of myxedema which ultimately develop, notably the condition of the skin and fur. This simple goiter apparently differs from the similar condition in man in the degree of the reaction that follows administration of iodine. When iodine is given to one having simple goiter not of very long standing the gland is involuted and the metabolic rate is restored to normal. When, however, iodine is given to rabbits having a large cabbage goiter metabolism rises far above normal, resulting in a marked loss in weight and finally death, as shown by Webster and Chesney (1) and confirmed by Marine (9). Apparently such rabbits do not possess the ability to control the secretion of thyroid hormone and limit it to the physiological needs when such a powerful stimulant to thyroid hyperplasia as that contained in cabbage is supplied.

From our experience with this type of goiter we estimated (by palpation) that the thyroids of our animals weighed between 0.5 and 1.0 gm. when the metabolism experiment was begun and the metabolic rate was approaching the myxedema level. After 10 mg. of iodine (as potassium iodide) were given, the glands would be involuted completely, the metabolism raised to the normal and perhaps beyond for a short time, after which, on continued cabbage feeding the thyroids again became hyperplastic and the metabolism decreased.

Our observations on rabbits agree with and complement those of Aub, Bauer, Heath, and Ropes in man. Not only is there a retention of calcium in myxedematous rabbits but also of mag-

nesium and phosphorus as well, while the other inorganic elements of the diet are relatively uninfluenced. When the thyroids of such animals are restored to the normal state by the administration of 2 to 10 mg. of iodine, the calcium, phosphorus, and magnesium balances are restored by an increased elimination of these elements. Continuation of the cabbage diet again results in thyroid hyperplasia and again we find a decrease in calcium, magnesium, and phosphorus elimination and consequent storing up of these elements.

SUMMARY

The relation of mineral metabolism of rabbits to the physiological state of the thyroid has been studied. In thyroid deficiency (caused by feeding rabbits with cabbage until a palpable goiter is produced) there is a retention of calcium, magnesium, and phosphorus. Converting the thyroid to the normal colloid-containing state by giving a small amount of iodine restores the balance of these elements by increasing the amounts excreted. The metabolism of sodium, potassium, chlorine, and sulfur is relatively uninfluenced by thyroid deficiency.

BIBLIOGRAPHY

1. Webster, B., and Chesney, A. M., *Bull. Johns Hopkins Hosp.*, **43**, 291 (1928).
2. Smith, G. F., and Ross, J. F., *J. Am. Chem. Soc.*, **47**, 1020 (1925).
3. Shohl, A. T., and Pedley, F. G., *J. Biol. Chem.*, **50**, 537 (1922).
4. McCrudden, F. H., *J. Biol. Chem.*, **7**, 83, 201 (1909–10); **10**, 187 (1911–12).
5. Fales, H. A., Inorganic quantitative analysis, New York, 214, 225 (1925).
6. Benedict, S. R., *J. Biol. Chem.*, **6**, 363 (1909).
7. Marine, D., and Williams, W., *Arch. Int. Med.*, **1**, 349 (1908).
8. Aub, J. C., Bauer, W., Heath, C., and Ropes, M., *J. Clin. Inv.*, **7**, 97 (1929).
9. Marine, D., unpublished data.

THE PREPARATION OF CRYSTALLINE ORNITHINE. THE PICRATES AND MONOSULFATES OF ORNITHINE*

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The decomposition of arginine, when heated with alkali, was first observed by Schulze and Steiger (1) who noted the formation of carbon dioxide and ammonia and of a strong organic base. The carbon dioxide and ammonia were soon shown (2) to be secondary products of decomposition, the primary product being urea, and Schulze and Winterstein (3) later identified the organic base as ornithine, employing for this purpose the dibenzoyl derivative, ornithuric acid. The free base was regenerated from this compound but could not be induced to separate from the solution. Another unsuccessful attempt to prepare crystalline ornithine was reported by Fischer and Raske (4) who worked with synthetic material secured by the treatment of β-vinylacrylic acid with ammonia.

So far as we have been able to discover, ornithine has not hitherto been secured in the crystalline form. Lysine, its next higher homologue, however, was recently obtained in crystals by Vickery and Leavenworth (5), and it therefore seemed probable that ornithine might also be crystallized if the proper conditions were ascertained. It was found, however, that d-ornithine would not separate from aqueous solution even when this was concentrated to a thick sirup and chilled for some time; only after the addition of liberal amounts of aldehyde-free alcohol and ether was the separation of a solid substance obtained. The details are given in the experimental part.

The preparation was a white powder which, under the microscope, was seen to consist largely of amorphous particles interspersed with tiny needles. Analysis[1] of this material, dried at 60°, showed the presence of C 43.49, H. 8.31, N 20.22, moisture 3.63, and ash 3.57 per cent. Corrected for ash and moisture, the composition was C 45.12, H. 8.62, N 20.98 per cent; the theory for ornithine is C 45.40, H 9.16, N 21.21 per cent. The *perfectly* white product was preserved by sealing into glass tubes but, on standing several months in the dark, it gradually acquired a faint orange-yellow color. It would seem from the present information, therefore, that free ornithine is not entirely stable. This is in marked contrast to the behavior of free lysine; a sealed tube of this material is apparently unchanged after standing in a dark place for 3 years.

<div align="center">EXPERIMENTAL</div>

Preparation of Arginase Powder—The press juice, obtained from finely ground pig livers, was warmed to 60°, cooled, filtered, and evaporated *in vacuo* at 45° to a sirup. The sirup was then frozen with solid carbon dioxide and transferred to a vacuum desiccator over sulfuric acid. Under a moderate vacuum the moisture was removed very rapidly and, after being thoroughly dried, the product could readily be reduced to powder in a mortar.

Decomposition of Arginine—Crystalline free arginine (6), in units of 3.106 gm. (equivalent to 0.5 gm. of ornithine nitrogen), was dissolved in 15 cc. of water and neutralized to pH 8.0 with sulfuric acid. The reaction was stabilized by the addition of 2 cc. of the appropriate phosphate buffer, and 0.2 gm. of the arginase powder was added. The mixture was stirred and then incubated at 37° for 2 to 3 days, toluene being used as a preservative. Under these conditions hydrolysis of 50 to 60 per cent of the arginine took place as indicated by the urea content of the solution; this was determined by the urease method.

Isolation of Ornithine as Picrate—The solution was acidified and the residual arginine was removed by precipitation in the usual way as the silver compound. The filtrate containing the ornithine was freed from reagents as far as possible and was concen-

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[1] Analysis by Research Service Laboratories, New York.

trated to a small volume. This solution was contaminated with many impurities derived from the enzyme preparation; purification of the ornithine by precipitation was therefore necessary. In some of the experiments a slight modification of the procedure of Bergmann and Zervas (7) was employed for this purpose. The solution, at a volume of 50 cc., was treated with 5 cc. of salicylaldehyde at 5°, the reaction being maintained strongly alkaline to phenolphthalein by the addition of barium hydroxide. The yellow precipitate of the barium salt of o-oxybenzylidene ornithine was filtered off, after standing in the cold for 48 hours, and was decomposed by warming with a small excess of dilute sulfuric acid. After filtration the solution was extracted with ether and the sulfuric acid was then quantitatively removed. The strongly alkaline solution was decolorized with norit, was concentrated to 10 to 15 cc., and treated with 3 gm. of picric acid dissolved in hot alcohol; it was then evaporated on the steam bath to incipient crystallization, which occurred at a volume of about 10 cc., and chilled for 24 hours. The crystalline ornithine dipicrate was filtered off and dried at 100°. The best yield secured under these conditions was 44 per cent; this was obtained in an experiment in which only 1.875 gm. of arginine had been taken. Much smaller yields (10 to 20 per cent) were more usual and recourse was therefore had to recovery of the ornithine by precipitation with phosphotungstic acid.

Kiesel (8) has thoroughly investigated the conditions under which this operation can best be conducted and our experience is entirely in accord with his. He found that the ornithine solution must be neutral to litmus and as concentrated as possible, the phosphotungstic acid must be added in concentrated aqueous solution in large excess, and ample time allowed for complete separation to take place. In our experiments the neutralized ornithine solution was concentrated to a sirup and treated with a concentrated phosphotungstic acid solution until no further precipitation occurred. After chilling for 48 hours, the precipitate was removed, was washed with phosphotungstic acid solution, and decomposed in acetone solution in the customary way. The solution of the base contained 74 per cent of the nitrogen in the crude ornithine fraction and yielded 54 to 56 per cent of ornithine as dipicrate, calculated on the arginine taken. This method

therefore appears more advantageous than that of Bergmann and Zervas.

Crystallization of Ornithine—d-Ornithine dipicrate (27.2 gm.) of m.p. 208° was decomposed by hot 10 per cent sulfuric acid, the picric acid was removed by filtration after chilling, and the residual traces by extraction with ether. The solution was then diluted to 500 cc. and neutralized to Congo red with pure barium hydroxide. The filtrate and washings from the barium sulfate were decolorized and concentrated to 300 cc., and the remaining sulfuric acid was quantitatively removed in an apparatus which prevented the access of carbon dioxide (for details see (5)). The filtered solution was then concentrated *in vacuo*, and in the absence of carbon dioxide, to about 6 cc., when it had become a thick, nearly colorless sirup. After standing at 5° for 4 days, no crystals appeared; 25 cc. of aldehyde-free alcohol were then added. The solution was rendered turbid but, on careful stirring, became clear. As no crystals had separated after chilling for some time, 2 cc. of absolute ether were added; this threw out a small, gray, flocculent precipitate which soon settled. At the end of 24 hours this was filtered off, was washed with a mixture of 10 cc. of alcohol and 5 cc. of ether, and discarded. The washings were allowed to fall into the main solution and 30 cc. of absolute ether were immediately added to this. A voluminous white precipitate separated and settled rapidly. After chilling 3 days, this was filtered off and was washed successively with 25 cc. of a mixture of equal parts of alcohol and ether and 10 cc. of ether; the sintered glass Gooch crucible, used as a filter, was then at once placed in a vacuum desiccator that contained sulfuric acid and a dish of 50 per cent sodium hydroxide solution. The thoroughly dried ornithine formed a snow-white cake which could readily be reduced to powder with a spatula. The product softened at 120° and melted to a colorless liquid at 140°. The specific rotation was $[\alpha]_D^{25} = +11.5°$ when observed in 6.5 per cent solution in water.

Picrates of Ornithine—The melting, or rather decomposition, points of the picrates of ornithine given in the literature show a conspicuous lack of agreement. The mono- and dipicrates of *d*-ornithine were therefore prepared and recrystallized until a constant decomposition point was obtained. Both salts darkened at about 200° and decomposed with gas evolution at 208°. A mixture of

equal parts of mono- and dipicrate decomposed at 194°. These observations were made with Anschütz thermometers; the correction for the emergent stem is therefore practically negligible.

Riesser (9) obtained a monohydrate of *d*-ornithine monopicrate that decomposed at 198-199°. This melting point is probably uncorrected and, furthermore, his preparation may have contained a small proportion of dipicrate; this would account for his lower observation. Kossel and Weiss (10) reported a preparation with a decomposition point of 203–204°.

d-Ornithine dipicrate crystallizes from concentrated aqueous solution in short, sulfur-yellow, anhydrous, rhombic prisms. It is best obtained by adding the theoretical quantity of picric acid dissolved in hot alcohol to the concentrated aqueous solution of the free base, and then evaporating to incipient crystallization; it can be best recrystallized from 50 per cent alcohol. A specimen obtained from ornithine prepared by the alkaline decomposition of arginine contained 18.7 per cent of nitrogen (theory 18.98 per cent) and 78.0 per cent of picric acid (theory 77.63 per cent).

d-Ornithine monopicrate crystallizes from concentrated aqueous solution in slender, orange-yellow needles. The salt is prepared by adding the theoretical quantity of picric acid to a concentrated aqueous solution of the free base and proceeding as in the preparation of the dipicrate. It is best recrystallized from 50 per cent alcohol, from which, in our hands, it separated in the anhydrous condition. Even the most carefully prepared specimens contained a few crystals of the dipicrate. A nearly pure sample contained 19.25 per cent of nitrogen (theory 19.39 per cent) and 64.04 per cent of picric acid (theory 63.44 per cent).

dl-Ornithine was prepared from triacetylarginine according to the method of Bergmann and Köster (11) and was converted to the dipicrate. This salt resembled the dipicrate of *d*-ornithine very closely. It decomposed at 208° and a mixture of the two in equal parts likewise decomposed at 208°. Riesser's preparation decomposed at 183-184° but contained 2.5 molecules of water of crystallization. Fischer and Raske (4) observed a melting point of 185° and Kossel and Weiss (10) found 195°. Like Kossel and Weiss' product our specimen was anhydrous.

Monosulfate of Ornithine—*d*-Ornithine dipicrate was decomposed by hot 10 per cent sulfuric acid, the picric acid was re-

moved, and the solution was neutralized to litmus with barium hydroxide. After filtration and decolorization the solution was concentrated to a sirup and treated with alcohol until turbid. The monosulfate separated, on standing in the cold for some hours, in a yield of 80 per cent of the quantity indicated by the nitrogen content of the solution. After being dried at 110°, the air-dry salt lost somewhat more than the equivalent of 1 molecule of water; it was probably a monohydrate. The dry product contained 15.3 per cent of nitrogen, theory 15.47 per cent. d-Ornithine mono-sulfate begins to darken at about 225° and decomposes with gas evolution at 234°. A specimen of d-ornithine monosulfate de-rived from ornithine prepared by the alkaline hydrolysis of ar-ginine had $[\alpha]^{23} = +6.0°$; a specimen obtained by enzyme hy-drolysis of arginine had $[\alpha]_D^{25} = +8.4°$. It is evident, therefore, that the preparation of ornithine by alkaline hydrolysis of arginine leads to a partially racemized product. To our knowledge d-ornithine monosulfate has not previously been described; it has many properties that make it a useful compound for work with this base.

dl-Ornithine monosulfate, prepared in the same manner from the dl-dipicrate already mentioned, darkened slightly from 225° and melted with gas evolution at 234°. Kossel and Weiss (12) described a preparation of this compound that decomposed at 213°.

SUMMARY

d-Ornithine can be brought to separate as a white, partially crystalline powder by the addition of liberal amounts of aldehyde-free absolute alcohol and absolute ether to a concentrated aqueous solution of the free base. It melts at 140° to a colorless liquid and has a specific rotation of $[\alpha]_D^{25} = +11.5°$. The product is not entirely stable even when sealed in glass containers and pre-served in the dark.

The monopicrate and the dipicrate of d-ornithine both decom-pose at 208°. The dipicrate of dl-ornithine likewise decomposes at the same temperature. The monosulfate of d-ornithine decom-poses at 234° and is a convenient salt of this base. It has a specific rotation of $[\alpha]_D^{25} = +8.4°$.

BIBLIOGRAPHY

1. Schulze, E., and Steiger, E., *Z. physiol. Chem.*, **11**, 43 (1886).
2. Schulze, E., and Likiernik, A., *Ber. chem. Ges.*, **24**, 2701 (1891).
3. Schulze, E., and Winterstein, E., *Z. physiol. Chem.*, **26**, 1 (1898).
4. Fischer, E., and Raske, K., *Ber. chem. Ges.*, **38**, 3607 (1905).
5. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **76**, 437 (1928).
6. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **76**, 701 (1928).
7. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **152**, 282 (1926).
8. Kiesel, A., *Z. physiol. Chem.*, **118**, 254 (1921).
9. Riesser, O., *Z. physiol. Chem.*, **49**, 210 (1906).
10. Kossel, A., and Weiss, F., *Z. physiol. Chem.*, **68**, 160 (1910).
11. Bergmann, M., and Köster, H., *Z. physiol. Chem.*, **159**, 179 (1926).
12. Kossel, A., and Weiss, F., *Z. physiol. Chem.*, **59**, 492 (1909).

THE DETERMINATION OF THE IODINE NUMBER OF LIPIDS

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A. Comparison of Hanus with Rosenmund-Kuhnhenn Method

Several methods for the determination of the iodine number of fats and oils have been used widely with satisfactory results in most cases.

Using pyridine sulfate dibromide as a halogenizing agent, Rosenmund and Kuhnhenn (1) published a new method which was used later for the micro determination of the iodine number by Dam (2) and Page (3).

In work on the nature of tumor lipids, in which the writer is engaged at present, a micro method for the iodine number was needed, and for this purpose a comparison between the Hanus, one of the widely used methods, and the recently reported Rosenmund-Kuhnhenn method was first attempted, with special reference to the time required for the reaction and the amount of the halogen used in the two methods.

The pyridine sulfate dibromide was prepared according to the Rosenmund-Kuhnhenn method; i.e., 16.5 cc. (16 gm.) of purified pyridine and 10.9 cc. (20 gm.) of concentrated H_2SO_4 were measured into separate flasks containing about 40 cc. of glacial acetic acid with cooling, then combined, 5 cc. (16 gm.) of bromine were dissolved in 40 cc. of glacial acetic acid added, and the whole volume was made to 2000 cc. with acetic acid.

The iodine number determinations were made by both the Hanus and Rosenmund-Kuhnhenn methods on the following lipids: oleic, ricinoleic, and linoleic acids, cholesterol, cholesterol palmitate, and cod liver oil. The amount of lipids used in each determination was 50 mg. dissolved in chloroform.

The ricinoleic acid was taken as an example of unsaturated oxy fatty acid. Its iodine number is 85. Linoleic acid which was used in this experiment showed a lower iodine number than the theoretical value due to the partial oxidation during the storage; however, for the purpose of comparing two methods, this sample was used without further purification. Cod liver oil served as an example of a mixture of various lipids.

TABLE I

Iodine Number of Various Lipids Determined by Hanus and Rosenmund-Kuhnhenn Methods

Lipids	Time of reaction	Iodine No.		Deviation from theory	
		Hanus	Rosenmund-Kuhnhenn	Hanus	Rosenmund-Kuhnhenn
	min.			*per cent*	*per cent*
Oleic acid................	15	92.8	89.7	3.1	−0.3
" "	30	92.8	89.7	3.1	−0.3
" "	60	92.3	90.0	2.6	0
" "	120	90.0	89.7	0	−0.3
Ricinoleic acid..........	30	86.7	85.0	2.0	0
Cholesterol..............	30	91.5	69.0	38.6	4.5
"	70	101.0	71.0	53.0	7.6
" palmitate....	30		41.9		2.7
" "	70	50.2	42.0	23.0	2.9
Cod liver oil.............	30	158.0	152.0		3.8*
" " "	70	158.0	152.0		

* Less than Hanus.

Cholesterol was prepared from gall-stones and the cholesterol palmitate was synthesized from cholesterol and palmitic acid. The melting point was 145° for the former, and 78° for the latter.

The results are shown in Tables I and II and in Fig. 1.

In Table I it may be seen that the Hanus method cannot be used for the determination of the iodine number of cholesterol and its ester since the values obtained by this method deviate greatly from the theoretical value, 66 for cholesterol and 40.8 for cholesterol palmitate, whereas the ones obtained by the pyridine dibromide method of Rosenmund and Kuhnhenn are very close to

the theory and little influenced by the time of reaction. In Table II it is definitely shown that the amount of halogen has much less influence on the iodine number of linoleic acid when pyridine dibromide was used. Schoenheimer (4) obtained good results in the determination of the iodine number of cholesterol by using Kaufmann's (5) method as well as the Rosenmund method.

TABLE II

Influence of Amount of Halogen on Determination of Iodine Number of Linoleic Acid by Hanus and Rosenmund-Kuhnhenn Methods

Amount of linoleic acid	Hanus method		Rosenmund-Kuhnhenn method	
	Excess of halogen	Iodine No.	Excess of halogen	Iodine No.
mg.	*per cent*		*per cent*	
10.0	1410	163.0	1330	159.0
20.0	640	166.1	610	160.0
40.0	277	163.5	258	159.0
60.0	160	157.8	137	160.5
80.0	100	153 5	78	160.5
100.0	69	146.0	43	159.5
120.0	46	140.5	20	158.5
150.0	27	129.5	4	146.0

1 per cent chloroform solution of linoleic acid was taken in amounts varying from 1 cc. to 15 cc. Chloroform was added to make up the volume of the aliquot to 15 cc. if necessary. To each of these 10 cc. of Hanus solution and to another series 20 cc. of pyridine bromide solution were added. These mixtures were left for 30 minutes at room temperature, then titrated with 0.1 N sodium thiosulfate solution.

The following conclusions are drawn from the results indicated in Tables I and II and Fig. 1.

1. For the determination of the iodine number of oleic and ricinoleic acids both the Hanus and the Rosenmund methods give the same value which is very near the theoretical.

2. In linoleic acid and cod liver oil the Hanus method gives 2 to 4 per cent higher values than the other.

3. The Rosenmund method is much less influenced by the amount of halogen than the Hanus method with linoleic acid.

4. The Hanus method is not suitable for the determination of the iodine number of cholesterol and its ester.

B. Micro Determination of the Iodine Number of Fatty Acids and Phospholipid in Tissues

As shown in the previous experiments the Rosenmund-Kuhnhenn method is preferable to the Hanus method for the determination of the iodine number of various lipids including cholesterol when fairly large amounts of material were available. It was now

ENMUND

THEORETICAL VALUE

60 90 120
TIME IN MINUTES
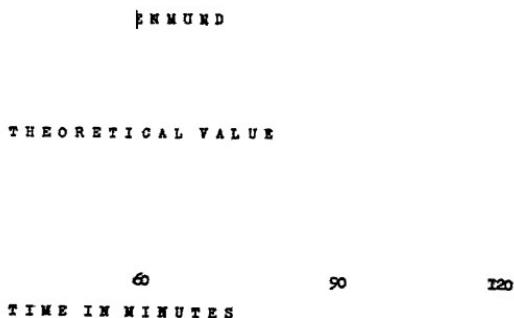
FIG. 1. Iodine number of cholesterol at different times of reaction

applied to the determination of the iodine number of small amounts of various lipids in the tissues of the animal body; *i.e.*, to the total fatty acids, phospholipid, and its fatty acids. The micro determination was found not only to give exactly the same results as the macro method in the standard lipid solutions, but also to be easy and simple to use.

Micro Determination of Iodine Number

Reagents

1. Diluted pyridine sulfate dibromide solution. The solution

prepared for the macro method is diluted to about 0.05 N with glacial acetic acid.

2. 10 per cent potassium iodide solution.

3. 1 per cent starch solution. To prevent bacterial action the starch solution is prepared in half-saturated KCl solution.

4. 0.02 N sodium thiosulfate solution.

Procedure—About 3 or 5 mg. of lipid dissolved in chloroform are added to 2 or 5 cc. of pyridine dibromide solution in a glass-stoppered Erlenmeyer flask and left for 15 minutes at the room temperature, after which 0.5 cc. of 10 per cent KI is added. The mixture is diluted with a small amount of distilled water and then titrated with 0.02 N sodium thiosulfate solution. (If the turbidity that occurs sometimes during the titration obscures the endpoint it can be removed by adding a small amount of glacial acetic acid.)

<div align="center">EXPERIMENTAL</div>

By the above procedures the iodine numbers of oleic, linoleic, and ricinoleic acids were determined and found to be 90 for oleic, 85 for ricinoleic, and 160 for linoleic acid, exactly the same as by the macro method. The amount of halogen to be used was investigated. The results are shown in Table III, in which it is apparent that the excess of halogen should be at least 25 per cent in the case of oleic acid and about 260 per cent in the case of linoleic acid.

Micro Determination of Iodine Number of Phospholipid

An aliquot of the tissue extract is evaporated to dryness on the steam bath and extracted with petroleum ether. Phospholipid is finally precipitated by acetone and $MgCl_2$ after Bloor's (6) method. After the residue of acetone is completely driven off by CO_2, the phospholipid precipitate is dissolved in chloroform and the insoluble substances, mostly $MgCl_2$, are centrifuged off. The clear chloroform solution is transferred to a volumetric flask and made up to volume with chloroform. It is advisable to make the chloroform solution so that 5 cc. of it contains about 3 or 4 mg. of phospholipid. An aliquot of the chloroform solution is used for the determination of the content of phospholipid by the oxidative method, and the same amount of aliquot is placed in a glass-stoppered Erlenmeyer flask to which an adequate amount of

pyridine dibromide solution is added to determine the iodine number. The following formula is used for the calculation.

$$\frac{a - b}{c} \times \frac{1.27}{5} = \text{I.N.}$$

TABLE III

Influence of Amount of Halogen on the Micro Determination of the Iodine Number of Oleic and Linoleic Acids

Fatty acid	Pyridine sulfate dibromide used	Excess of halogen	Iodine No.
mg.	*cc.*	*per cent*	
Oleic			
2.0	2.0	548	86.5
4.0	2.0	218	89.0
6.0	2.0	109	89.0
8.0	2.0	58	88.5
10.0	2.0	26	89.0
12.0	2.0	9	85.8
Linoleic			
2.0	5.0	780	159.0
4.0	5.0	340	159.0
5.0	5.0	255	158.0
6.0	5.0	199	156.0
8.0	5.0	125	156.0
10.0	5.0	84	152.0
Linoleic*			
5.0	2.0	56	141.5
5.0	2.0	56	141.2
5.0	4.0	195	150.5
5.0	4.0	198	149.0
5.0	5.0	262	153.0
5 0	5.0	258	155.4

* This solution of linoleic acid was made 2 days before the determinations were made. The iodine number of this solution was 154, showing a lower value than the freshly prepared solution which had the iodine number 159 as indicated in the table.

in which a is the number of cc. for the blank titration; b, the number of cc. of 0.02 N $Na_2S_2O_3$ needed for the titration; and c, the number of gm. of phospholipid.

To obtain the iodine number of the phospholipid fatty acids the

phospholipid is separated, dissolved in about 10 cc. of hot alcohol, and saponified by boiling with sodium ethylate for 20 minutes. The content of the flask is brought to dryness with the help of a stream of CO_2 on the steam bath, acidified with dilute sulfuric acid, and extracted with petroleum ether. The petroleum ether is evaporated and the remaining fatty acids are taken up with chloroform. The chloroform solution is used for the determination of the iodine number.

_ The fatty acids separated from the phospholipid acids by the above procedure are determined oxidatively, the factor 3.60 being used for the calculation as in the determination of other fatty acids. The results are shown in Table IV.

TABLE IV

Iodine Number of Phospholipid and Phospholipid Fatty Acids in Animal Tissues Determined by Micro Estimation

Rat tissues	Phospholipid in aliquot	Iodine No.	Tissues	Phospholipid fatty acids in aliquot	Iodine No.
	mg.			*mg.*	
Liver........	6.80	119	Mouse liver	2.99	140
Kidney......	2.67	98	" fat tissue	2.08	116
Skin........	2.33	59	Rat tumor	3.27	136
Muscle......	4.27	119			
Heart.......	3.67	122			

The tissues were extracted with alcohol and ether in the proportion of about 1 gm. of moist tissue in 100 cc. of solvent.

The values described in Table IV are the averages of each four determinations, the mean deviation in each case being between 2 and 6 per cent. As far as the standard solution of lipids, like oleic or linoleic acid, in which the content of the lipid is known, is concerned, the deviation was less than 2 per cent. The accuracy of this method depends upon the determination of the lipid content by the oxidative method since the titration values after the treatment with pyridine dibromide gave exactly the same duplicates. Attention must be called to the protection of phospholipid or fatty acids against oxidation during the procedures. The manipulations should be carried out without any delay and the contact of the lipids with air is to be prevented by the use of CO_2.

Using chloroform as the final solvent of phospholipid instead of moist ether as described in Bloor's original method has some advantages, since chloroform dissolves phospholipid as well as moist ether, but dissolves much less $MgCl_2$ so that this substance is removed by centrifuging and moreover the chloroform solution can be directly used for the iodine number determination.

Determination of Iodine Number of Total Fatty Acids in Tissues

By total fatty acids are meant the fatty acids set free by saponifying and subsequent acidification of the tissue extracts. The fatty acids are extracted with petroleum ether according to the description of Bloor's oxidative method for total lipid determination. The petroleum ether is evaporated and the remaining

TABLE V

Iodine Number of Fatty Acids in Tissue Extracts Determined by Micro Estimation

| Rat tissue | Content in aliquot | | Iodine No. |
	Fatty acids	Cholesterol	
	mg.	*mg.*	
Liver........................	3.63	0.21	137
Kidney.......................	3.07	0.36	122
Skin.........................	10 80	0.29	85
Muscle.......................	10.65	0.19	104

substances are extracted with chloroform completely. The chloroform solution is made to a certain volume of which one part is used for the determination of the content by oxidative method and the other for the iodine number of the fatty acids.

Since cholesterol cannot be separated by the above procedures, this must be considered in the calculation of the iodine number of total fatty acids. The iodine number of cholesterol can be determined by the pyridine dibromide method and the value is 69 as noted previously. If pyridine dibromide is titrated with 0.02 N $Na_2S_2O_3$ after cholesterol was halogenized, 1 mg. of cholesterol would require 0.27 cc. of that sodium thiosulfate solution, therefore the formula for the calculation of the iodine number of total fatty acids is

$$\frac{(a - b) - 0.27 \times d}{c} \times \frac{1\ 27}{5} = \text{I.N.}$$

where a represents cc. of the blank titration; b, cc. of 0.02 N thiosulfate solution needed for the titration; c, the number of gm. of fatty acids; and d is the number of mg. of cholesterol in the same aliquot.

The alcohol-ether extracts of some tissues of rats were used as the materials for this experiment. In Table V the average values of each four determinations are described.

Thus, by the use of the pyridine dibromide solution of Rosenmund and Kuhnhenn and the oxidative micro method of lipids of Bloor, the iodine number of lipids in small amounts of tissues can be determined.

The writer is deeply indebted to Professor W. R. Bloor for his advice and helpful criticism of this work.

BIBLIOGRAPHY

1. Rosenmund, K. W., and Kuhnhenn, W., Z. Untersuch. Nahrungs- u. Genussmittel, 46, 154 (1923).
2. Dam, H., Biochem. Z., 152, 101 (1924); 220, 158 (1930).
3. Page, I. H., Biochem. Z., 223, 445 (1930).
4. Schoenheimer, R., Z. physiol. Chem., 192, 78 (1930).
5. Kaufmann, H. P., Z. Untersuch. Lebensmittel, 51, 3 (1926).
6. Bloor, W. R., J. Biol. Chem., 82, 273 (1929); 77, 53 (1928).

THE CATALYTIC EFFECT OF FERRICYANIDE IN THE OXIDATION OF UNSATURATED COMPOUNDS BY OXYGEN

By G. PAYLING WRIGHT, JAMES B. CONANT, and S. E. KAMERLING

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It has been shown recently (Wright, 1931) that oleic acid in sodium carbonate solution absorbs oxygen rather rapidly if potassium ferricyanide is also present, whereas in the absence of this reagent the autoxidation is too slow to be measured in the usual apparatus. At first sight it would seem that the catalytic effect of the ferricyanide is due to a cyclic series of reactions which may be indicated as follows:

(1) Ferricyanide + oleic acid → oxidation products + ferrocyanide

(2) Ferrocyanide + O_2 → ferricyanide

We have carried out a series of experiments to test this mechanism and find that it does not represent the course of the reaction. Ferrocyanide in sodium carbonate solution does not absorb oxygen at an appreciable rate (measured in the usual Warburg apparatus). Furthermore the reduction of ferricyanide by oleic acid *in the absence of oxygen* is very slow indeed. This is shown in Curve 3, Fig. 1. Even after 192 hours in nitrogen at 37°, a sodium carbonate solution initially 0.064 M in oleic acid and 0.1 M in ferricyanide was found to contain an amount of ferrocyanide corresponding to only an 8 per cent reduction of the ferricyanide. If we assume that 2 mols of ferricyanide react with 1 mol of oleic acid, only about 6 per cent of the oleic acid was oxidized after 192 hours by the action of ferricyanide alone. As a matter of fact it seems probable that the interaction of the two substances is even

less than this figure as an examination of Curve 3, Fig. 1, shows a rather rapid reduction of the first 6 per cent of the ferricyanide and a very slow action from this point on; a small amount of oxidizable impurity may be responsible for the initial 5 per cent reduction of the ferricyanide.
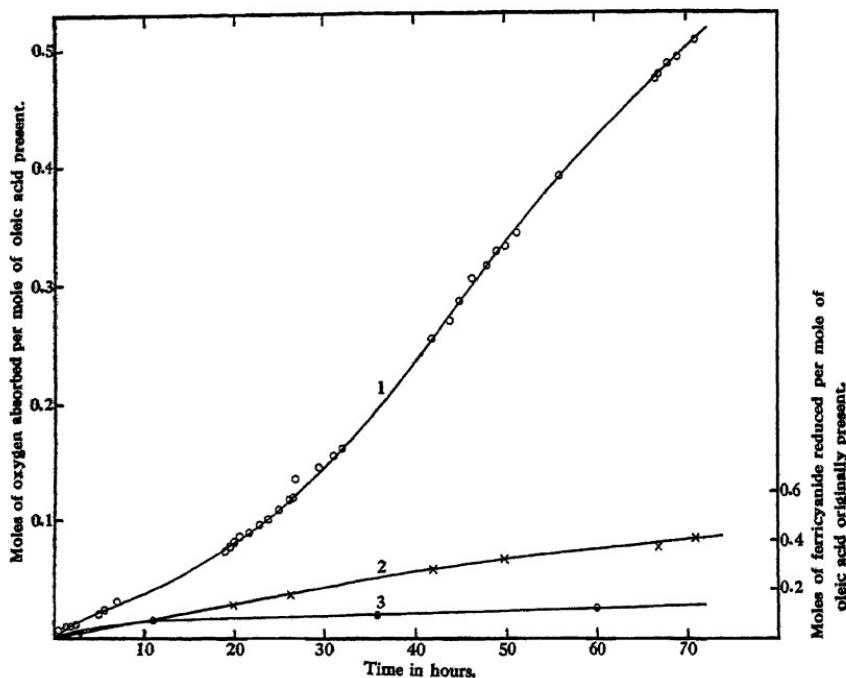
Fig. 1. The rate of oxygen absorption of oleic acid and the rate of ferricyanide reduction at 30°. The absorption vessel contained 2 cc. of 0.5 per cent sodium carbonate solution 0.032 M in oleic acid and 0.125 M in ferricyanide. Curve 1, oxygen absorption of oleic acid; Curves 2 and 3, reduction of ferricyanide in air and nitrogen, respectively.

It is thus easy to establish that a cyclic process is not responsible for the catalytic action of the ferricyanide, since neither Reaction 1 nor Reaction 2 in the suggested scheme proceeds at a rate sufficient to account for the oxygen absorption.

In this connection it should be pointed out that the statement on p. 767 of an earlier paper (Wright, 1931) must be qualified. The sentence "That potassium ferricyanide is able to oxidize

unsaturated fatty acids is definitely shown by its oxidation of oleic acid dissolved in sodium carbonate," must be amended by substituting as the initial phrase "potassium ferricyanide and oxygen together are able."

A study of the reduction of ferricyanide by oleic acid *in the presence of oxygen* has shown that during the catalyzed autoxidation the ferricyanide is reduced. This is shown by Curve 2, Fig. 1. The experiment was carried out with the same solutions and under the same conditions as those employed in the oxygen absorption experiments reproduced by Curve 1. In these experiments and those previously mentioned, the amount of ferrocyanide formed was determined by the electrometric titration of an aliquot sample with potassium molybdicyanide. This method has proved rapid and reliable in this laboratory in other work; the accuracy is within ± 2 per cent.

The limit of the oxygen absorption by oleic acid in the presence of ferricyanide appears to be 1 mol of oxygen per mol of acid. This limit was reached after about 200 hours at 40°.

In view of the facts presented above, it seems to us likely that the autoxidation of oleic acid in the presence of ferricyanide is a chain reaction. The function of the ferricyanide may be imagined as being similar to that of the cupric ions in the oxidation of sulfite solutions which according to Haber (1931) act catalytically by initiating a series of chain reactions. Some preliminary experiments which we have performed indicate that sodium phenolate, isobutyroin, and to a less extent benzyl alcohol are negative catalysts for the induced oxidation of oleic acid by ferricyanide. This investigation is being continued and expanded to include the study of the action of other oxidizing agents in inducing the autoxidation of unsaturated compounds; we hope to report later results which will decide definitely whether or not a chain reaction is involved. Since circumstances have prevented one of us from continuing this work, we have been prompted to publish this preliminary account of a study of the mechanism of the reaction.

BIBLIOGRAPHY

Haber, F., *Naturwissenschaften,* **19,** 450 (1931).
Wright, G. P., and Arthur, B., *J. Biol. Chem.,* **90,** 757 (1931).

THE ACID-BASE BALANCE IN ANIMAL NUTRITION

IV. THE EFFECT OF LONG CONTINUED INGESTION OF ACID ON REPRODUCTION IN SWINE, RATS, AND RABBITS

BY ALVIN R. LAMB AND JOHN M. EVVARD

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Paper I of this series (1) reports the addition of N sulfuric acid solution to a ration of cereals and meat meal tankage which was fed to swine. Amounts up to 500 cc. of N acid per animal per day were fed over a period of about 10 months. The number of animals was only two in each lot, however, and the young produced were not reared. Metabolism studies (2) and further acid-feeding experiments (3) gave additional evidence that there was no unfavorable effect upon the growth or well being of the animals if the amount of acid fed was not more than 500 cc. of N solution per day. The possibility, however, of serious effects of such large amounts of acid upon reproduction has been obvious, and has not been excluded by the data thus far reported. The present paper is, therefore, concerned with the effects of long continued ingestion of mineral acid upon reproduction through successive generations. The vigor, appetite, and condition of the animals (swine) and their performance in reproduction over a period of $3\frac{1}{2}$ years have been observed; two groups which were fed and cared for exactly alike, except for the feeding of the sulfuric acid, were compared. Similar data on rats and rabbits are also reported.

The literature contains many papers on the mechanism of acid-base equilibrium under various abnormal or pathological conditions. Some studies have been reported on the effects of acid feeding on the skeletal and other tissues and on the composition of the blood. Few attempts have been made to superimpose a considerable acidity upon a normal ration under normal feeding conditions.

Salmon (4) has reported failure of growth in the second generation of rats which received a good basal ration plus 1.5 per cent of acid phosphate. Better results were obtained when 0.5 per cent of sodium bicarbonate was added to the ration. The toxic effect, however, of the fluorine which is always found in acid phosphate was disregarded in his report. Burns (5) reported changes in the composition of the bones and soft tissues of rats and rabbits which were fed hydrochloric acid. The rats, however, were fed only 9 weeks, and the rabbits 17 to 35 days. Addis, MacKay, and MacKay (6) have reported that no degenerative changes occurred in the kidneys of rats receiving a good synthetic diet plus 2 per cent of calcium chloride, which was assumed to be equivalent to the addition of 14.4 cc. of n hydrochloric acid solution per 100 gm.

Citation and discussion of earlier papers on the subject appear in Paper III of this series (3).

In most of the many acid-feeding experiments in the literature, either acid-forming diets which are deficient nutritionally are fed, or acid is given as such in amounts which cause death or suspension of growth within a relatively short time. No effort is made in this type of experiment to feed a satisfactory ration plus an amount of acid which can be tolerated by the animal over a considerable time, with the purpose of testing the efficiency of the neutralization mechanism which must function when normal but potentially acid diets are fed. These experiments are not continued long enough to determine whether or not there is a physiological adjustment to the acid diet. Erroneous conclusions are drawn, analogous to those formulated by Bunge on the basis of the early effect of the ingestion of potassium salts on the excretion of sodium. The animal organism has a remarkable ability to adjust itself to changed conditions. Whether or not this adjustment will eventually cause harm to the organism can be determined only by continuing the experimental regimen through several reproductive cycles.

EXPERIMENTAL

Experiments with Swine

The experiments with swine were started with two lots of four females each, at an initial weight of about 125 pounds each. The

ration fed was ground corn 70 parts, meat meal tankage 5 parts, corn germ meal 10 parts, and alfalfa meal 15 parts. Salt in block form was given *ad libitum*. The feed mixture was thoroughly moistened with water, and the sulfuric acid given to Lot 2 was added as a N solution and mixed with the moist feed in the trough. Lot 1 served as the control, and received the same amount of the ration plus the same amount of added water.

Each animal in Lot 2 received 100 cc. of N acid solution per day for 30 days, and 200 cc. per day for the next $7\frac{1}{2}$ months, at which time the acid was increased to 250 cc. of N solution per day. At this time the four litters of young which had been farrowed in Lot 2 were from 2 to 3 weeks of age. This amount of acid was continued without interruption until the young were weaned. The total number of pigs in Lot 1 was forty, of which thirty-five were rated as good and five as weak. In Lot 2 there were twenty-seven good pigs and three weak pigs.

At the end of the suckling period five representative female pigs were selected from each lot, to be continued on the same ration. The pigs in Lot 2 were immediately started at a level of 50 cc. of N acid per animal per day. After 30 days the amount of acid was doubled, and after another 30 days it was increased to 200 cc. After 60 days at this level the acid was increased to 300 cc. per animal per day. This is 5 times as much acid as the potential acidity of the fattening ration of corn, meat meal tankage, and wheat middlings which we used in our earlier work. This level was maintained up to and throughout the first gestation and suckling periods of these sows, or a total of 7 months at the 300 cc. level.

One sow in each lot failed to breed and two sows in Lot 1 (control) lost their pigs at birth or soon after, leaving only two litters in Lot 1 and four litters in Lot 2. The total number of pigs farrowed in Lot 1 was nineteen, of which fourteen were good normal pigs. In Lot 2 the total was twenty-two, of which twenty were of normal vigor.

At the end of the suckling period of these young, four representative females were selected from each lot, to be continued under the same treatment in their respective lots, as before. The acid allowance per animal was 100 cc. of N solution during the first 70 days, 200 cc. per day during the next 60 days, and 250 cc. per

day during the next 7 months, or until the young produced had been weaned. In this case, four sows in Lot 1 produced young, but only two litters were farrowed in Lot 2. This may have been due to failure of impregnation as the records show only one service for each sow. Similar cases of failure to reproduce are occasionally observed in sows kept under restricted conditions without pasture, as these were. In the preceding generation it has been noted that the control lot suffered more than did the acid-fed lot. It is not possible, however, to rule out completely at this point the possibility of some unfavorable effect of the ration on reproduction. The total number of pigs farrowed in Lot 1 was thirty-one, of which one was weak and one born dead. In Lot 2, the total number of pigs was seventeen in two litters, all of which were rated good.

After these young were weaned, the same sows were continued 6 months longer under the same experimental regimen, until the next litters had been farrowed and weaned. At this time four sows in Lot 1 and three sows in Lot 2 produced and raised young. The totals in Lot 1 were twenty good pigs, two weak, and two dead; and in Lot 2, twenty-eight good pigs, and three which were dead at birth. No differences in performance, appetite, or vigor were apparent between the two lots in greater degree than is commonly observed between any two groups of hogs kept under similar conditions. In the case of this last group of sows, the feeder noted that Lot 2 had generally a better appetite than Lot 1. This was reflected in the comparative rates of growth.

The animals were fed according to appetite throughout the experiment, since the matter of growth on this type of ration plus sulfuric acid has been fully tested in preceding experiments (1, 3). The gain in weight of these animals was normal and satisfactory, both during the suckling and growth periods and during gestation.

Postmortem Observations

At the end of the suckling period of the second litters of these sows the experiment was closed, after forty-three months (December 5, 1919, to July 1, 1923). Two new generations of animals had been produced, and four complete reproductive cycles completed.

Data on the composition of the bones of representative animals of the three generations are shown in Table I. Macroscopic

observations of the viscera and skeleton were made, no differences outside of usual individual variations and no pathological lesions at all being found.

TABLE I

Analyses of Bones of Representative Animals (Per Cent of Air-Dry Sample)

	Moisture	Ether extract	Ca	Mg	Na	K	P	Cl	S
Sternum and rib; control; 1st generation	8.75	10.30	13.95	0.11	0.48	0.65			
Acid-fed; 1st generation	8.40	15.00	13.16	0.14	0.44	0.44			
Radius and ulna; control; 1st generation	7.90	18.95	15.70	0.26	0.38	0.31			
Acid-fed; 1st generation	7.70	20 20	15.80	0.33	0.36	0.29			
Rib, radius, and ulna; control; 2nd generation			16.56	0.27	0.39	0.35	8.19	0.11	1.22
Acid-fed; 2nd generation			17.33	0.40	0.41	0.35	8.62	0 11	1.24
Skull and teeth; control; 2nd generation			17.55	0.51	0.50	0.34	8.82	0.49	1.14
Acid-fed; 2nd generation			17.82	0.53	0.45	0.36	8.92	0.42	1.06
Skull, humerus, scapula, radius, ulna (combined); acid-fed; 3rd generation	5.13	17.70	18.45	0.45	0.48	0.32	8.93	0.33	0.83
Control; 3rd generation	5.06	20.00	17.08	0.50	0.47	0.35	8.68	0.26	0.84
Acid-fed; 3rd generation	5.65	14.35	18.80	0.45	0.48	0.37	9.02	0.42	0.83

Experiments with Rats

A ration consisting mainly of materials carrying a potentially acid ash plus a highly acid salt mixture was fed to rats over a period of 16 months. The ration was as follows:

Salt Mixture

Yellow corn.............. 33	$CaH_4(PO_4)_2 \cdot H_2O$...........1.0
Oats.................... 25	$CaHPO_4$..................0.8
Barley.................. 25	NaCl.....................0.3
Alfalfa meal............ 6	$MgSO_4 \cdot 7H_2O$...............0.2
Wheat embryo........... 2.5	KH_2PO_4.....................0.2
Casein.................. 5	$K_2S_2O_7$.....................1.0
Salts................... 3.5	KI.......................Trace
100	3.5

The potential acidity of the whole ration (average analyses for the cereal grains therein) was calculated to be 15 cc. of N solution per 100 gm. of ration. This corresponds with an intake of 1.5 to 2.0 cc. of N acid per mature rat per day. Four rats, three of them males, were started on this ration on March 24, 1921, at an average weight of 61 gm. The young produced were carried along on the same ration, all making normal growth. Young were produced with normal frequency by the original female and by her descendants, with from four to thirteen young per litter. Mature animals were discarded and the young continued. After 11 months some of the animals began to appear somewhat rough coated. The ration was then fortified with 2 per cent cod liver oil, although there were no clinical signs of rickets. The appearance of the rats, however, improved after the cod liver oil addition.

At the end of the experiment the females and their litters were in excellent condition, and their appearance was fully as good as that of the rats in the regular stock colony. No possible ill effect could be seen in the appearance, appetite, growth, or reproduction of these rats.

Experiments with Rabbits

Many acid-feeding experiments were carried on with rabbits over a period of 4 years, with rations of oats, alfalfa, casein, and standard sulfuric acid solution. The proportions of the components of the ration were varied, but kept in such amounts that a slight excess of acid was produced when the ration was digested. Rabbits are rather sensitive to acid diets, as has been reported by Winterberg (7), Funk (8), Lyman (9), and others. None of these workers has, however, fed rations which were of normal physical character for the rabbit and complete from a nutritional standpoint. In our experiments the rabbits grew and reproduced normally if the excess acid per day was not over 5 cc. of N solution.

The performance of one rabbit (female) may be cited. Started at a weight of 1155 gm., this rabbit reached a weight of 3650 gm. in 6 months, when a normal litter of young was produced, followed by four other litters in the next 12 months. Although still in good condition, the rabbit finally died in parturition from uterine hemorrhage after 24 months on the acid ration. The numerous progeny of this doe were placed on the same acid ration for varying

lengths of time, some but not all of them making as good records as the doe.

The greater part of the acid was excreted in the urine as acid phosphates, giving an acid titer varying between 25 cc. and 45 cc. of 0.1 N solution for 24 hours. Compared with rats and swine, much less ability to elaborate ammonia to be used for the neutralization of acid was seen. Frequent analyses of the urine corroborated the acid character of the ration and indicated that the ability of the animals to combine ammonia with the acid possibly tended to increase. The ratio $\dfrac{NH_3\text{-}N}{\text{total N}}$ in the urine varied from 0.5 to 1.0 per cent for the original doe mentioned above and varied between 1.0 and 3.4 per cent for the young.

When the net total acid intake, actual and potential, was raised above 5 cc. of N solution per rabbit per day, the animals quickly lost appetite and soon died if the acid was continued. Large amounts of acid as such in the ration were tolerated (25 cc. of N solution), but a large net acid intake could not be borne by these animals. The net amount tolerated is much less per unit of body weight than that taken by swine and rats.

SUMMARY

Swine were fed for three generations ($3\frac{1}{2}$ years) on a ration of yellow corn, tankage, corn germ meal, and alfalfa meal plus 200 to 300 cc. of N sulfuric acid solution per day. No deleterious effects on growth, well being, or reproduction were observed in the group receiving the acid. No unfavorable effects on the composition of the bones of representative animals were found.

Three generations of rats were produced with no apparent ill effect and with optimum reproduction on a satisfactory grain, casein, and acid salts ration which contained a total potential acidity of 15 cc. of N acid solution per 100 gm. of feed.

Rabbits tolerated a ration containing a net acidity up to 5 cc. of N solution per animal per day, the basal ration being optimum in composition and physical character for rabbits. When the net acid allowance was increased above 5 cc. of N solution, the rabbits soon succumbed. These animals excreted nearly all of the acid as acid phosphates in the urine, having only slight ability to mobilize ammonia for neutralization of the acid.

BIBLIOGRAPHY

1. Lamb, A. R., and Evvard, J. M., *J. Biol. Chem.*, **37**, 317 (1919).
2. Lamb, A. R., and Evvard, J. M., *J. Biol. Chem.*, **37**, 329 (1919).
3. Lamb, A. R., and Evvard, J. M., *Iowa Agric. Exp. Station, Research Bull. 71* (1921).
4. Salmon, W. D., *Science*, **62**, 420 (1925).
5. Burns, C. M., *Biochem. J.*, **23**, 860 (1929).
6. Addis, T., MacKay, E. M., and MacKay, L. L., *J. Biol. Chem.*, **71**, 157 (1926-27).
7. Winterberg, H., *Z. physiol. Chem.*, **25**, 202 (1898).
8. Funk, C., *J. Biol. Chem.*, **25**, 409 (1916).
9. Lyman, J. F., and Raymund, B., *J. Biol. Chem.*, **39**, 344 (1919).

CATALYTIC OXIDATION OF d-GLUCOSE AND RELATED SUGARS BY OXYGEN IN THE PRESENCE OF IRON PYROPHOSPHATES

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Extensive research on the oxidation of the carbohydrates has been reported. Previous work has been carried out in relatively strong alkaline solution and at elevated temperatures in most cases, hence the conditions of these experiments were far removed from body processes. Nef[1] and his students used about 6 equivalents of alkali in an initial concentration of 5 per cent sodium hydroxide, and air and hydrogen peroxide as oxidizing agents. Later Anderson[2] used Fehling's solution and other metallic oxidizing agents. Evans[3] and his students have used alkaline permanganate, silver oxide, and copper acetate. Power and Upson[4] used air and calcium hydroxide, and Jensen and Upson[5] carried out oxidations by means of cupric ion in the presence of sodium carbonate. Thus the trend has been toward conditions approaching those in the body through the use of weaker alkalies and lower temperatures. Doubtless the nearest approach in this direction has been made by Spoehr[6] who showed that an iron pyrophosphate solution, made faintly alkaline by the addition of alkali phosphates and activated by air, readily oxidizes at room temperature monosaccharides, disaccharides, polyhydric alcohols, and some of the

[1] Nef, J. U., Ann. Chem., 357, 214 (1907); 403, 204 (1914).

[2] Anderson, E., Am. Chem. J., 42, 401 (1909).

[3] Evans, W. L., Chem. Rev., 6, 281 (1929). (Contains a bibliography of the work of Evans and his students.)

[4] Power, M. H., and Upson, F. W., J. Am. Chem. Soc., 48, 195 (1926).

[5] Jensen, F. W., and Upson, F. W., J. Am. Chem. Soc., 47, 3019 (1925).

[6] Spoehr, H. A., J. Am. Chem. Soc., 46, 1494 (1924). Spoehr, H. A., and Smith, J. H. C., J. Am. Chem. Soc., 48, 236 (1926).

hydroxy dibasic acids, to give carbon dioxide as one of the end-products. This paper gives the results of more comprehensive work which is a continuation of that begun by Spoehr and which has been carried out at his suggestion.

EXPERIMENTAL

Chemicals—The catalyst was prepared according to Spoehr[6] except for slight changes in the amount of water used and in the procedure. To 16.8 liters of distilled water were added 804 gm. of sodium pyrophosphate (Mallinckrodt, $Na_4P_2O_7 \cdot 10H_2O$, c.p.). When this was dissolved, 120 gm. of ferrous sulfate (Merck, $FeSO_4 \cdot 7H_2O$, c.p.) were added and shaken frequently until solution was complete. Then 2040 gm. of disodium phosphate (Mallinckrodt, $Na_2HPO_4 \cdot 12H_2O$, c.p.) were added and solution effected by passing carbon dioxide-free air into it, and the bubbling of the air continued until the green color had faded to a light yellow.

Pfanstiehl's glucose, fructose, arabinose, and mannitol and Eastman's mannose and α-methylmannoside were used. The α-methylglucoside, tetramethylglucose, gluconic acid, tetra-ethyl-α-methylglucoside, tetramethyl-α-methylmannoside, α-methylarabinoside, and arabonic acid were prepared in this laboratory.

Apparatus—A diagram of the apparatus finally adopted is shown in Fig. 1. The oxygen from the cylinder was led through a pressure-reducing valve into a sulfuric acid drying train, then successively through calcium chloride, soda-lime, and ascarite, the two latter serving to remove traces of carbon dioxide. The purified oxygen was then led through a distilled water wash bottle before entering the distributor shown in Fig. 1, from which its flow to the oxidation towers was regulated by means of screw clamps.

The oxidation chamber consisted of a glass tube 75 cm. long and 33 mm. in internal diameter, sealed to a Jena glass filter funnel fitted with a fritted glass plate. The oxygen passing through this porous plate was divided into a stream of very fine bubbles. The gases from the oxidation towers were led through the sulfuric acid wash bottles shown in Fig. 1, then through dehydrite tubes

to insure complete drying, and finally through the weighed ascarite tubes for the absorption of the carbon dioxide.

In each experiment four control tubes were used. To guard against back absorption, the weighed ascarite tubes were all surmounted by smaller ascarite tubes, and these were protected by inverted test-tubes containing calcium chloride supported by cotton plugs.
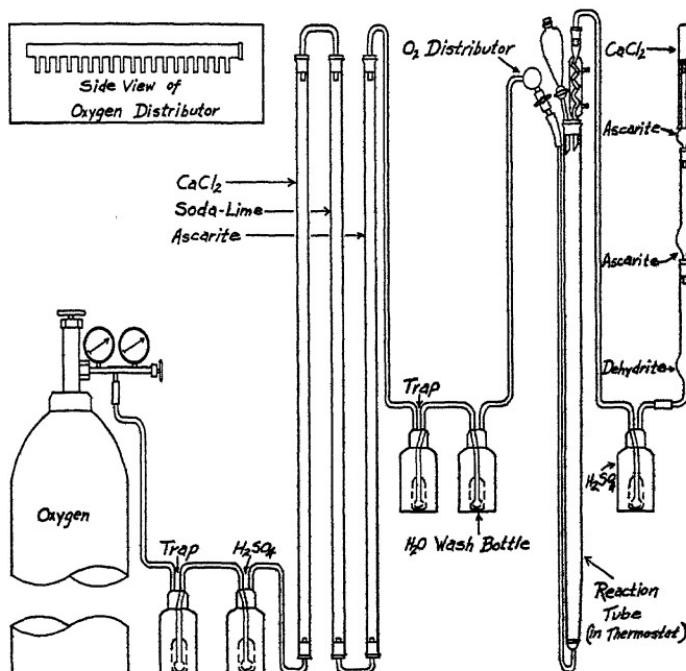
FIG. 1. Apparatus for the catalytic oxidation of d-glucose and related sugars by oxygen in the presence of iron pyrophosphates.

Procedure—To start an experiment, $\frac{1}{180}$ mol of the sugar was weighed out, dissolved in, and diluted with the catalyst solution to 4550 cc. 500 cc. of this solution were then transferred to each of eight oxidation towers. The towers were then connected with the absorption train, and the flow of oxygen regulated at a speed which assured no darkening of the solution. The temperature of the thermostat was maintained at about 48°.

Determination of carbon dioxide was made by direct weighings

TABLE I
Carbon Dioxide Produced at Different Intervals of Time

Time in days	Carbon dioxide, mM								C returned as CO_2, per cent	
	¼	1	2	5	8	12	16	23	16	23
Arabinose	1	13	23	36	44	52	58	123	20.8	44.6
α-Methylarabinoside	0	0	0	0	0	0	0	28	0	8.4
Arabonic acid	1	31	47	56	61	63	64	104	23.0	37.4
Glucose	1	23	37	50	56	61	66	159	19.8	47.7
α-Methylglucoside	0	0	0	3	9	15	21	103	5.4	26.5
Gluconic acid	1	4	14	37	48	57	63	135	18.9	40.5
Tetramethylglucose	0	0	0	0	0	0	0		0	
Tetraethyl-α-methylglucoside	0	0	0	0	0	0	0		0	
Fructose	19	53	71	97	111	120	126	142	37.8	42.6
Mannose	1	30	44	57	65	73	79	167	23.7	50.1
α-Methylmannoside	0	0	0	3	9	18	24	85	6.1	21.8
Mannitol	0	0	0	0	1	8	24	144	7.2	43.2
Glycerol	0	6	15	16	16	16	16	18	9.6	10.8
Glyceric acid	1	3	3	3	3	3	3	3	1.8	1.8
Glycolic "	1	3	3	3	3	3	3	3	2.7	2.7

The temperature was maintained at about 48° for 16 days, then at about 5° for 7 days.

TABLE II
Oxidation Products Formed at Different Intervals of Time

Time in days	CO_2		Volatile acid		Total acid		Aldoses, cc. N iodine solution reduced		
	16	23	16	23	16	23	0	16	23
	mM	mM	mM	mM	mM	mM			
Arabinose	58	123	29	29	51	75	92	44	8
α-Methylarabinoside	0	28	0	18	0	50	1	2	
Arabonic acid	64	104	11	12	21	39	2	2	2
Glucose	66	159	21	21	60	98	91	46	9
α-Methylglucoside	21	103	17	34	42	103	2	13	7
Gluconic acid	63	135	11	16	31	56	3	6	5
Tetramethylglucose	0		0		0		88	90	
Tetraethyl-α-methylglucoside	0		0		0		1	1	
Fructose	126	142	34		84		5	4	
Mannose	79	167	23	22	64	92	79	38	8
α-Methylmannoside	24	85	18	26	37	90	1	11	9
Mannitol	24	144	18	25	37	97	2	23	7
Glycerol	16	18	18		24		1	2	
Glyceric acid	3	3	0		10		0	1	
Glycolic "	3	3	0		2		0	0	

of the lower ascarite tubes at regular intervals; the average gains in weight per tube are given in Tables I and II.

Total acid was found by adding the proper amount of phosphoric acid, then distilling under reduced pressure at about 60° into standardized alkali, followed by back titration; and the aldose value was obtained by means of the Cajori iodine titration.[7]

From the data given in Tables I and II, it is observed that all but two of the fifteen compounds give some carbon dioxide under the conditions of these experiments. If the rate of carbon dioxide formation is taken as a measure of the ease of oxidation, these compounds may be arranged in the order of increasing stability to give the series fructose, mannose, glucose, arabonic acid, arabinose, gluconic acid, α-methylmannoside, α-methylglucoside, mannitol, glycerol, glyceric acid, glycolic acid, α-methylarabinoside, tetramethylglucose, and tetramethyl-α-methylglucoside. Arabonic acid appears to be out of its logical place, and the ease with which α-methylmannoside and α-methylglucoside give carbon dioxide is indeed surprising.

DISCUSSION

If glucose is taken as a type for these oxidation reactions of the simple sugars, and Nef's[8] use of the theory of enolization is applied, the following four mechanisms may be postulated.

1. The aldehyde group may undergo direct oxidation to give the corresponding sugar acid. This in turn may enolize and undergo further oxidation to give either carbon dioxide, water, and arabonic acid or a keto acid. If the keto acid appears as an intermediate, it might on further oxidation yield carbon dioxide, water, and arabonic acid or through decomposition give carbon dioxide and the next lower sugar.

$$
\begin{array}{c}
H \cdot CO \qquad HO \cdot CO \quad HO \cdot C \cdot OH \quad HO \cdot CO \cdot OH + HO \cdot CO \cdot Rx \\
| \qquad\qquad | \qquad\qquad \| \qquad \nearrow \\
H \cdot C \cdot OH \rightarrow H \cdot C \cdot OH \rightleftharpoons C \cdot OH \\
| \qquad\qquad | \qquad\qquad | \qquad \searrow \\
Rx \qquad\qquad Rx \qquad\qquad Rx \qquad HO \cdot CO \quad CO_2 + HOH + HO \cdot CO \cdot Rx \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad | \nearrow \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad CO \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad | \searrow \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad Rx \quad CO_2 + H \cdot CO \cdot Rx
\end{array}
$$
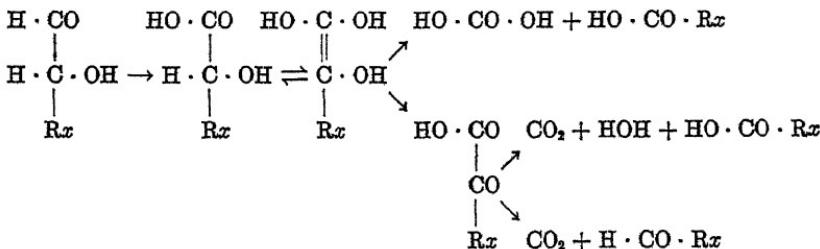
[7] Cajori. F. A.. *J. Biol. Chem.*. **54.** 617 (1922).

2. Tautomeric shift of the aldehyde group under the influence of the alkali into the 1,2-enediol, followed by subsequent oxidation, might yield either formic acid and arabonic acid, or carbon dioxide, water, and arabonic acid. But here again the keto acid may be formed as a possible intermediate product which, by loss of carbon dioxide, might give the next lower sugar.

$$
\begin{array}{lll}
\text{H} \cdot \text{CO} & \text{H} \cdot \text{C} \cdot \text{OH} & \text{H} \cdot \text{CO} \cdot \text{OH} + \text{HO} \cdot \text{CO} \cdot \text{R}x \\
\ | & \ \| & \nearrow \\
\text{H} \cdot \text{C} \cdot \text{OH} \rightleftharpoons \text{C} \cdot \text{OH} & \searrow \\
\ | & \ | & \text{H} \cdot \text{CO} \rightarrow \text{HO} \cdot \text{CO} \quad \text{CO}_2 + \text{HOH} + \text{HO} \cdot \text{CO} \cdot \text{R}x \\
\text{R}x & \text{R}x & \ | \qquad\qquad | \nearrow \\
& & \text{CO} \qquad\quad \text{CO} \\
& & \ | \qquad\qquad | \searrow \\
& & \text{R}x \qquad\quad \text{R}x \quad \text{CO}_2 + \text{H} \cdot \text{CO} \cdot \text{R}x
\end{array}
$$
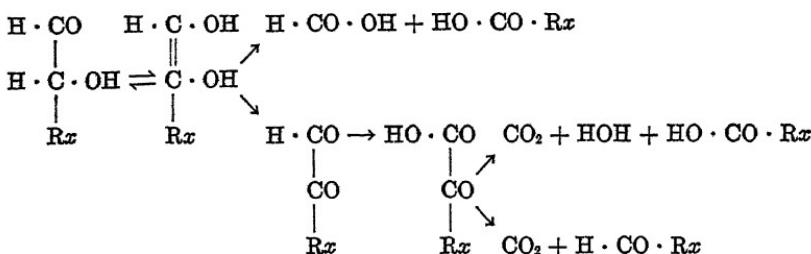
3. Regardless of the nature of the reactions at the aldehyde group, there is the possibility of oxidation at the primary alcohol group. This might be expected to follow the oxidation mechanism of a primary alcohol group in alkaline solution to give the corresponding aldehyde. Subsequent oxidation at this group might be expected to follow one of the mechanisms outlined above.

$$
\begin{array}{lllll}
\text{R}z & \text{R}z & \text{R}z & \text{R}z & \text{R}z \\
\ | & \ | & \ | & \ | & \ | \\
\text{H} \cdot \text{C} \cdot \text{OH} \rightleftharpoons \text{H} \cdot \text{C} \cdot \text{OH} \ \text{or} \ \text{H} \cdot \text{C} & \rightarrow & \text{H} \cdot \text{C} \cdot \text{OH} & \rightarrow & \text{H} \cdot \text{C} \cdot \text{OH} \\
\ | & \ | & \ \| & & \ | & & \ | \\
\text{H}_2\text{C} \cdot \text{OH} \ \ \text{H} \cdot \text{C} \rightleftarrows & \text{H} \cdot \text{C} \cdot \text{OH} \ \text{H} \cdot \text{C(OH)}_2 & \text{H} \cdot \text{CO}
\end{array}
$$
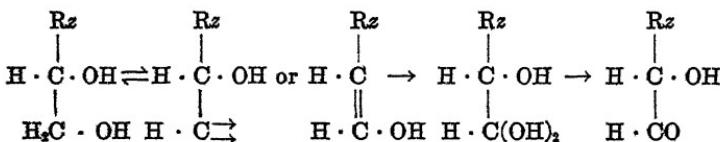
4. There remains the possibility of a combination of any or all of the above reactions, all proceeding simultaneously.

Interpretation of Results

From a consideration of the data given in Tables I and II with respect to their applicability to the above postulates, the following conclusions seem to be warranted.

The activity of the catalyst as an oxidizer is greatly increased by an increase in temperature. This is clearly shown by a comparison of the results obtained at the end of 16 days at 48° with those obtained 7 days later at a temperature of above 98°.

All the sugar acids experimented with gave some carbon diox-

ide.   This suggests that the initial oxidation may give the monobasic sugar acid, as outlined in postulate (1).   However, glucose and glycerol give carbon dioxide much more readily than the corresponding acids.   Furthermore, Spoehr[6] found that saccharic acid gave practically no carbon dioxide.   Thus it seems that the rate of carbon dioxide formation cannot be wholly dependent upon the initial oxidation of the aldehyde group to the acid group. On the other hand, arabonic acid does give carbon dioxide much more readily than does arabinose.

The glucoside, mannoside, and arabinoside were oxidized.   Thus hydrolysis of the methoxy group must occur, or else oxidation must proceed from the primary alcohol group.   The possibility of hydrolysis of the methoxy group is suggested by von Fellenberg's work[9] on the determination of this group when present as glucoside, ester, or ether.   He reports that glucoside or ester methoxy group may be removed by a 2 per cent solution of sodium hydroxide at 80–90°.   The effect of the phosphate solution on the glucoside with respect to hydrolysis of the methoxy group is reserved for a later paper.

The results obtained from glycerol and mannitol clearly show that this catalyst can oxidize a primary alcohol group of a polyhydroxy compound to carbon dioxide under the conditions of these experiments.   Thus oxidation by this catalyst may proceed from both ends of the sugar molecule, starting no doubt at the end of least resistance and then proceeding from either or both ends as determined by the electronic structure of the molecule.

The data are best interpreted by use of postulates (2) and (3). In case there is no free aldehyde group, the primary alcohol group may be first attacked and oxidized to the aldehyde, followed by enolization and subsequent oxidation to the keto aldehyde, then to the keto acid which is reported by Honig and Tempus[10] to be an intermediate in the oxidation of glucose.

[9] von Fellenberg, T., *Biochem. Z.*, **85**, 44 (1918).

[10] Honig, M., and Tempus, F., *Ber. chem. Ges.*, **57**, 787 (1924).

This may be represented as follows:

$$
\begin{array}{lll}
\text{H} \cdot \text{CO} & \text{H} \cdot \text{C} \cdot \text{OH} & \text{H} \cdot \text{CO} \cdot \text{OH} + \text{HO} \cdot \text{CO} \cdot \text{R}x \quad \text{(Reaction A)} \\
\quad | & \quad \| & \qquad\qquad\qquad\nearrow \\
\text{H} \cdot \text{C} \cdot \text{OH} \rightleftharpoons & \text{C} \cdot \text{OH} & \qquad\qquad\qquad\searrow \\
\quad | & \quad | & \text{H} \cdot \text{CO} \quad \text{HO} \cdot \text{CO} \quad \text{CO}_2 + \text{HOH} + \text{HO} \cdot \text{CO} \cdot \text{R}x \\
\quad \text{R}x & \quad \text{R}x & \qquad | \qquad\qquad | \;\nearrow \\
& & \qquad \text{CO} \;\; \rightarrow \;\; \text{CO} \\
& & \qquad | \qquad\qquad | \;\searrow \\
& & \qquad \text{R}x \qquad\quad \text{R}x \quad \text{CO}_2 + \text{H} \cdot \text{CO} \cdot \text{R}x \\
& & \qquad\qquad\qquad\qquad\qquad\quad \text{(Reactions B)}
\end{array}
$$
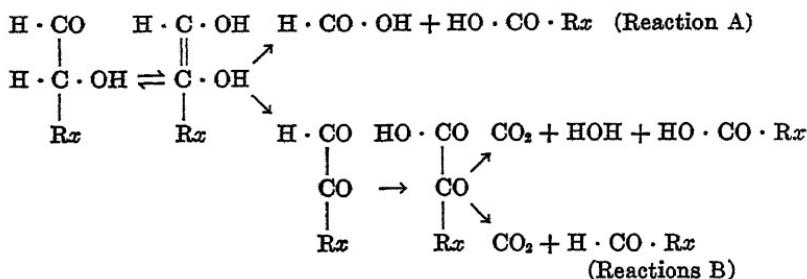
With this mechanism in mind, consider the 1, 2-enediol.　Under the influence of an oxidizing agent, it is apparent that the chances of both bonds breaking at the same time to give formic acid and the next lower sugar acid are far less than the chances of but one of the bonds breaking.　It but one bond breaks, the keto aldehyde may appear as an intermediate product, which may undergo enolization and subsequent oxidation to the keto acid.　The keto acid may then oxidize to carbon dioxide, water, and the next lower sugar acid, or decompose to give carbon dioxide and the next lower sugar.　Graphically, the decomposition to carbon dioxide and the next sugar may be represented as follows:

$$
\begin{array}{ccccccc}
\text{O}- \;\; +\text{H} & \text{O} & \text{H} & \text{OH} & \text{OH} & \text{OH} \\
& \| & + & | & | & | \\
& ++ & - & | & | & | \\
\text{O}^-_{\;} \;\; +\overset{+}{\text{C}}+ & -\overset{++}{\text{C}}- \;\; +\overset{+}{\text{C}}+ & -\overset{-}{\text{C}}- \;\; +\overset{-}{\text{C}}+ & -\overset{-}{\text{C}}- \;\; +\text{H} \\
& + & + & + & + \\
& \text{OH} & \text{H} & \text{H} & \text{H}
\end{array}
$$
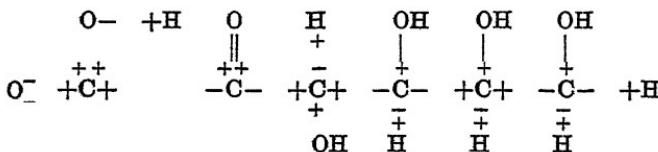
This shows the carbon atom of the carboxyl group to be completely positive, thus permitting the loss of carbon dioxide without disturbing the rest of the molecule.　There seems to be a similarity in this respect to malonic ester and to orthophthalic acid.　Since formic acid is not oxidized under the conditions of these experiments, the carbon dioxide must come from another source.　The above formula suggests that the carbon dioxide may be accounted for by assuming that a part of the intermediate keto acid decomposes to give a molecule of carbon dioxide and a molecule of the next lower sugar.　Such a hypothesis explains the fact that at the end of 16 days the aldose concentration is about half of the initial aldose concentration, and the additional fact

that after 7 days more of oxidation at 98° the aldose concentration is still about one-tenth that of the original.

If we accept the hypothesis that the enediol[1] is the active intermediate in these reactions, then it must be that fructose forms this unstable linkage much more readily than does mannose, glucose, or arabinose.   Consider these formulas:

$$
\begin{array}{cc}
\text{O : C} \cdot \text{H} & \overset{\displaystyle \text{H}}{\underset{}{|}} \\
| & \text{HO} \cdot \text{C} \cdot \text{H} \\
\text{H} \cdot \text{C} \cdot \text{OH} & | \\
| & \text{C : O} \\
\text{HO} \cdot \text{C} \cdot \text{H} & | \\
| & \text{HO} \cdot \text{C} \cdot \text{H} \\
\text{R}x & \text{R}x
\end{array}
$$
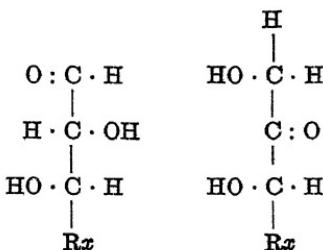
In the case of the aldoses, there is but a single H atom on the α-carbon atom that can migrate to the terminal carbonyl group with the consequent formation of the 1,2-enediol; whereas in the case of the ketose sugars there are 2 H atoms on the terminal carbon atom, either of which may migrate to the α-carbonyl oxygen to give the same enediol. This would suggest that, in the absence of steric hindrance, fructose should form the 1,2-enediol twice as readily as glucose. In addition, fructose may form the 2,3-enediol with the same ease that glucose forms the 1,2-enediol. Hence with both the 1,2- and the 2,3-enediols, it appears that fructose has three chances to enolize, whereas glucose, under the same conditions, has but one chance to form an enediol.

SUMMARY

In conclusion, it appears (a) that the rate at which the sugars are oxidized under these conditions is a function of the temperature, (b) that the phosphate effects enolization of the sugars, (c) that the rate of oxidation is a function of the rate of enolization, (d) that the aldehyde is first formed, if not already present, then enolizes and oxidizes either to formic acid and the next lower sugar acid or to the keto aldehyde, (e) that the keto aldehyde, if formed, is oxidized to the keto acid of Honig and Tempus,[10] and (f) that the keto acid appears to decompose to give carbon dioxide and the next lower sugar, or oxidizes in part to give carbon dioxide, water,

AMINO ACID METABOLISM

I. THE RELATIVE RATES OF AMINO ACID DISAPPEARANCE AND UREA FORMATION

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INTRODUCTION

The current theories of protein catabolism usually postulate the oxidative or hydrolytic deamination of amino acids. The nitrogen is believed to be converted into urea, but little, if anything, is definitely known about the intermediate steps in its formation. If these are but brief, if the intermediate compounds have only an ephemeral existence, the decrease in amino nitrogen to the basal level following the injection of amino acids should keep pace, quantitatively, with the increase in urea formation. If, on the contrary, these nitrogenous intermediates are significant in duration, it should be possible to demonstrate their existence by comparing the rate of urea formation with the rate of amino acid disappearance in injected animals.

With this end in view we have approached the problem by applying to groups of animals which had received amino acids by subcutaneous injection the methods of tissue analysis. It was our hope that analysis of the whole animal would yield results which necessarily escape observation when attention is confined to the composition of the excreta only.

EXPERIMENTAL

Young albino rats between 120 and 200 gm. in weight were used. The animals were of a standard strain from our own colony and had been maintained for many generations on a uniform and constant diet.[1] 1 week before an experiment nine or ten animals

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[1] Finely cracked wheat, 2600 gm.; finely ground oats, 2600 gm.; yellow corn-meal, 2600 gm.; flaxseed meal, 1000 gm.; whole milk powder, 1000 gm.; alfalfa meal, 500 gm.; powdered dry yeast, 200 gm.; bone meal, 150 gm.; sodium chloride, 50 gm.; cod liver oil, 50 cc.; lettuce, daily.

of the same sex and closely alike in weight were selected and caged together.   36 hours before injection the group was transferred to a cage with a coarse screen bottom and deprived of food.   In most of the experiments an environmental temperature of 27–29° was maintained during the fasting and experimental periods.   On termination of the fast each rat was weighed, its bladder emptied, and a measured volume of the solution promptly injected beneath the skin of the thoracic and lumbar regions.   The animal was then placed in an individual metabolism cage, consisting of a 6 inch evaporating basin covered by a coarse screen and a large inverted funnel.   A trace of mercuric chloride was added to the basin to inactivate fecal urease.

Of the nine or ten animals in the group all but two or three received an injection of the amino acid under investigation.   The remainder, which served as controls, received an equal volume of 0.9 per cent sodium chloride.   The quantity of amino acid administered was calculated on a basis of 0.3 or 0.4 gm. of N per kilo of body weight.   It was dissolved in distilled water, made up to a suitable volume, and so injected that the heaviest member of the group received 5 cc.   The concentration of amino acid nitrogen in the injection fluid was checked by analysis (Van Slyke method). d-Glutamic and l-aspartic acids were administered as the monosodium salts.

The times of injection were so arranged as to leave intervals of at least 30 minutes between killings.   The members of the group were then killed in prearranged order so that intervals of about 0, 1, 2, 3, 5, 7, and 12 hours occurred between the time of injection and the time of killing.   The controls were usually analyzed after 1, 7, and 12 hours.

The rest of the procedure, including the treatment of the carcass and preparation of the extract for analysis, has been described elsewhere (1).   Amino acid nitrogen was determined by the method of Kiech and Luck (2) and urea by the volumetric xanthydrol method of Allen and Luck (3).

Results

To permit the plotting of values obtained from several experiments upon the same chart and the preparation of a single composite curve, it was necessary to apply certain corrections to the experimentally determined values.

In some groups the control animals exhibited an amino nitrogen content of only 33 to 35 mg. per 100 gm. of body weight.   In other groups the base-line was as high as 47 or 48.   Although the cause of this group variation is not known with certainty, it was clearly necessary for purposes of comparison to reduce the amino nitrogen values for all groups to a common base-line.   The level selected was 42.2 which represents the average amino nitrogen content, in mg. per 100 gm. of body weight, of 55 control animals.

A second correction was then applied to make due allowance for differences in the quantities of amino acid administered.   The amount injected was as low, in some series, as 0.30 gm. of N per kilo and in others as high as 0.40.   In most cases an injection level of 0.35 gm. of N per kilo was maintained, and in those series in which different quantities were injected, a correction was applied to restore all values to the 0.35 level.   The correction was calculated as a function of the amount of amino acid deaminized. To illustrate the method employed the calculations in a typical series are presented in Table I.

In plotting the urea values similar corrections were necessary. One factor requiring consideration was found to be the curious difference existing between various groups of rats in the basal level of urea formation.   By control determinations on 55 animals we have found that rats fasted for 36 hours have a urea content of 62.0 mg. per 100 gm. of body weight.   After 1 hour in a control run the value rises to 66.4 mg., after 7 hours to 92.8 mg., and after 12 hours to 114.8 mg.,[2] indicating a basal rate of urea formation of 4.4 mg. per 100 gm. of body weight per hour.[3]   The average urea base-line is therefore a straight line having its origin at 62 mg., and ascending in 12 hours to 114.8 mg. per 100 gm. of body weight.   We have noticed several series, however, which presented interesting departures from this general form of behavior, the base-line for the group being quite definitely above or below the one described, the latter being a composite average for the controls of all the series.   In one group the urea base-line

[2] These values include the urea excreted during the experimental period.

[3] Cori and Cori (4) found that rats fasted between 24 and 48 hours excreted 3.18 ± 0.30 mg. of N per 100 gm. per hour.   If it is assumed that 80 per cent of the urinary nitrogen is in the form of urea, this would represent the formation of 5.4 mg. of urea per 100 gm. per hour.

TABLE I

Calculation of Amino Nitrogen and Urea Values. Glycine, Series ●——●

Animal No.*	0_c	1_c	2_c	3_c	5_c	7_c	12_c	0	1_c	3_c	7_c	12_c
Amino nitrogen												
As determined, *mg. per 100 gm.*	81.0	80.2	73.2	71.3	57.3	64.2	63.5	42.0	47.8	44.5	48.3	44.0
Group correction factor†	−3.1	−3.1	−3.1	−3.1	−3.1	−3.1	−3.1					
Corrected values, *mg. per 100 gm.*	77.9	77.1	70.1	68.2	54.2	61.1	60.4					
Quantity deaminized (*a*), *per cent*	8.5	10.5	28.4	33.4	69.1	51.5	53.3					
Second correction factor (*x*)‡	−3.7	−3.6	−2.9	−2.7	−1.2	−1.9	−1.9					
Final values, *mg. per 100 gm.*	74.2	73.5	67.2	65.5	53.0	59.2	58.5					
Urea												
As determined, *mg. per 100 gm.*	58.4	69.6	65.5	78.8	106.3	114.2	171.1		51.9	81.5	78.3	102.3
Group base-line; computed 0 values§	58.4											
" correction factor‖	+7.8	+7.8	+7.8	+7.8	+7.8	+7.8	+7.8					
Corrected values, *mg. per 100 gm.*	66.2	77.4	73.3	86.6	114.1	122.0	178.9					
Urea increase (*d*), ¶ *mg. per 100 gm.*		4	8	12	25	35	60					
Second correction factor (*y*)**	0	−0.5	−0.9	−1.4	−2.9	−4.0	−6.9					
Final values, *mg. per 100 gm.*	66.2	76.9	72.4	85.2	111.2	118.0	172.0		47.5	68.3	47.5	49.5

* The letter "e" signifies an experimental animal, "c" a control. The figure indicates the time in hours after injection.

† The group correction factor is 42.2 minus the average of the control values in the preceding row, 42.2 being the average obtained on 55 control animals. The purpose of the group correction factor is to reduce the values of all series to a common base-line.

‡ Correction factor $(x) = \dfrac{100 - a}{100}(35 - b)$ where b = concentration of amino acid injected in mg. of amino N per 100 gm. of body weight. In this particular case $b = 39$. The purpose of this correction is to restore the values of all series to the same injection level; *viz.*, 35 mg. of amino N per 100 gm. of body weight.

§ Calculated by subtracting from the 1_0, 3_0, 7_0, and 12_0 values, 4.4, 13.2, 30.8, and 52.8, representing the amount of urea formed by control animals in 1, 3, 7, and 12 hours respectively.

‖ The group correction factor is 62 minus the average of the values in the preceding row, 62 being the mean zero value obtained on fourteen control animals. The purpose of this correction is to reduce all values to a common base-line.

¶ Difference between plots of values in the preceding row and the composite base-line for controls.

** Correction factor $(y) = \dfrac{35 - b}{35} d$, where b is the concentration of amino acid injected in mg. of amino N per 100 gm. of body weight. The purpose of this correction is to restore the values of all series to the same injection level; *viz.*, 35 mg. of amino N per 100 gm. of body weight.

was found to lie 15.5 mg. below the composite average for all series, and in another 16.1 mg. above,—the rate of urea formation, nevertheless, remaining in the vicinity of 4.4 mg. per 100 gm. of body weight per hour.   The cause of this group divergence is not yet clear although it is presumably due to differences in the nutritional condition of the different groups.   Several groups employed in the earlier experiments were fasted 24 hours instead of 36, and, in addition, the environmental temperature was not rigidly controlled except in the later series.   It is conceivable that sex differences between groups may also be involved.

In consequence of this shifting base-line we have found it necessary to apply a correction by which the values obtained from any given group are adjusted by a constant to raise or lower the entire series to a common base-line.   The constant used in this correction has been computed by averaging the zero and control values less the urea formed in the animal's basal metabolism.   If the average so obtained differs from 62, the accepted zero value for the base-line, the difference is added to or subtracted from each value in the series.   Table I illustrates the nature of the calculation.

As it is not feasible to print all the seventeen tables of which Table I is but a single representative, we have compiled instead all of the original uncorrected values in Table II and presented the data requisite for calculation of the group correction factors in Table III.   By the use of this material the final values may be calculated.

For convenience in interpretation the results have been reexpressed as percentage changes in deamination and urea formation, and set forth graphically in Fig. 1.   The final corrected values referred to in the preceding paragraph were first plotted.   Smooth curves were then drawn, from which the values presented in Fig. 1 were readily obtained.   The preliminary graphs used in the preparation of the figure will gladly be sent on request to any interested.

DISCUSSION

As might be expected, striking differences are to be observed in the amino acid disappearance curves.   It was nevertheless surprising that the amino acid which underwent most complete

Amino Nitrogen and Urea Values As Determined (Uncorrected)

The number heading the column in each section indicates the time of killing the animal (hours after injection). The values are expressed in mg. per 100 gm. of body weight.

Amino acid	Series	Amino N							Urea						
		0	1	2	3	5	7	12	0	1	2	3	5	7	12
Glycine	●—●	81.0	80.2	73.2	71.3	57.3	64.2	63.5	58.4	69.6	65.5	78.8	106.3	114.2	171.1
"	○—○	80.6	72.1	67.4	63.5	61.3	58.6	56.4	44.9	58.8	71.5	70.0	89.7	121.2	142.0
" *	△—△	81.6	80.0	73.0	70.0	58.8	63.6	53.4 / 54.8	60.0	73.6	109	133	147	158	204 / 219
dl-Alanine	○—○	76.6	65.8	55.5	47.5	50.6	43.5	40.1	76.6	73.0	108.3	120.6	136.3	124.6	166.0
d-Glutamic acid	○—○	76.8	67.5	65.5	64.0	51.6			36.9	49.9	67.9	95.7	109.5		
"	×—+	61.2	60.7	50.2	47.7	45.2									
" *	△—△	84.5	76.0	73.7	74.8	65.5	63.4	58.5	64.5	85.0	86.6	121.4	104.0	124.0	
" *		81.0	77.5	75.6	70.0	67.0	56.3								
l-Aspartic acid	○—○	77.4	68.2	64.0	62.1	64.0	64.1	67.5	64.7	51.7	84.0	101.8	104.8	128.8	154.5
"	×—×	68.5	67.8	68.2	60.8	60.8	60.4	64.6	54.1	73.1	106.0	125.0	149.9	
l-Tyrosine	●—●	61.5		60.5	62.7	64.8	63.5	61.0	60.4	78.2	83.5	92.6	85	112.4	

Amino acid	Series	Amino N							Urea						
		0	1	2	3	5	7	10	0	1	2	3	5	7	10
dl-Alanine	●—●	68.8	61.2	49.6	43.2	41.5	43.1	40.8	52.9	75.5	80.6	101.0	122.0	113.8	121.0
l-Aspartic acid	●—●	71.8	72.0		64.5	62.1	59.3	63.2 (8)	66.5	84.6		108.2	119.2	118.0	165.7 (8)

Amino acid	Series	Amino N							Urea						
		0	1	2	3	5	7	11	0	1	2	3	5	7	11
Glycine*	+—+	81.0	80.0	70.2	67.6	57.5	60.0	52.7 / 59.0	43.6	64.6	110	107	141	151	173 / 177
" '	×—×														

Amino acid	Series	Amino N							Urea						
		0	¾	1½	2¼	4	6	8	0	¾	1½	2¼	4	6	8
d-Alanine	●—●	72.0	64.2	50.8	64.9	47.8	59.3	48.4	64.2	71.5	96.4	124	131.2	134	139.8
d-Glutamic acid	●—●	72.5	68.6	63.2				51.9	63.1	60.0	96.4				151

Amino acid	Series	Amino N						Urea						
		0	¾	1¾	2¼	4	6	0	¾	1¾	2¼	4	6
dl-Alanine	×—×	74.4	69.4	60.6	55.0	52.8	45.7	62.0	71.3	84.5	86.6	106.7	121.7

TABLE III

Correction Factors for All Series

Amino acid	Series	Quantity injected (b)	Amino N — Control values	Amino N — Group correction factor†	Urea — Group base-line Computed 0 values	Urea — Group correction factor‖
Glycine	●—●	39	42, 47.8, 44.5, 48.3, 44.0	−3.1	58.4, 47.5, 68.3, 47.5, 49.5	+7.8
"	○—○	38	41.6, 37.0, 43.4, 41.5	+1.3	44.9, 46.4, 46.7, 45.7	+16.1
"	+—+	39	42.0, 44.8, 43.4	−1.2	43.6, 46.6, 82.2	+4.5
"	△—△	40	41.6, 49.5, 45.0	−3.2	60.0, 69.2, 103.2	−15.5
dl-Alanine	●—●	32	36.8, 35.0, 33.8, 35.3	+7.0	52.7, 50.6, 51.2, 43.7	+12.4
"	○—○	36.5	40.1, 47.5, 42.4, 41.3, 41.0	−0.3	76.6, 59.4, 60.4, 45.7, 65.8	+0.4
"	×—×	34	40.4	+1.8		0
d-Alanine	●—●	36	36.0, 37.2, 36.1	+5.8	64.2, 63.6, 67.4	−3.1
d-Glutamic acid	●—●	40	32.5, 33.3, 36.4	+8.1	63.1, 73.8	−6.5
"	○—○	31	45.8	−3.6	36.9, 45.5	+20.8
"	×—×	27–32		0	64.5	−2.5
"	+—+	39	45.5, 41.0, 45.0, 43.6	−1.6		
"	△—△	36	45.0, 45.8, 46.0, 43.5	−2.9		
l-Aspartic acid	●—●	35	36.8, 38.4, 34.4	+5.7	66.5, 92.2, 52.2	−8.3
"	○—○	35	42.4, 42.4, 44.5, 46.7	−1.8	64.7, 73.1, 61.2, 51.2	−0.6
"	×—×	31.5	37.0, 40.5	+3.4	51.3, 61.9, 74.2	−0.5
l-Tyrosine	●—●		39.1, 41.0, 44.9, 45.2	−0.4	60.4, 65.7, 62.0, 57.2, 63.2	0

For explanation

catabolism, as judged by the amino nitrogen results, was the racemic mixture of *d*- and *l*-alanine. Within 12 hours the amino
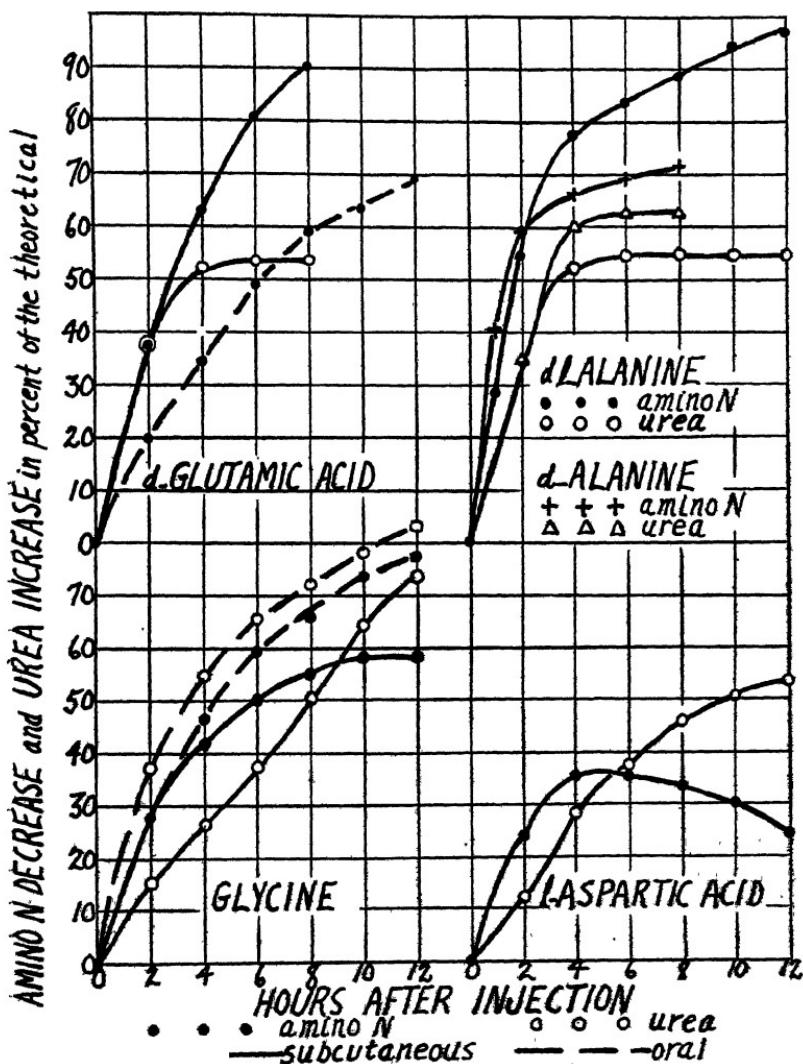
FIG. 1. The relative rates of deamination and urea formation

nitrogen content had fallen back from the high postinjection level to that of the control animals. This fact convinces us that

both d- and l-alanine are capable of utilization by the animal, and indicates that under the conditions of these experiments there is no loss of either isomer by excretion. At the same time, it is necessary to observe the curious cessation in urea formation that sets in when but little more than half of the expected amount has formed. This cannot mean that of the two isomers d-alanine alone is capable of urea formation, for the investigations of Abderhalden and Schittenhelm (5), Levene and Meyer (6), and Wilhelmj and Bollman (7) convince us that l-alanine, in part at least, gives rise to urea, while the research of Ringer and Lusk (8) on sugar formation from amino acids in the phlorhizinized animal shows that racemic alanine suffers almost complete conversion to glucose. Quantitative differences in the rates of metabolism of the two isomers are, however, to be expected and under certain conditions the contrast in utilization may be quite marked (9–11).

In the light of the thesis that we propose to advance in subsequent paragraphs we are inclined to recognize somewhat of a divorce, under certain conditions, between amino nitrogen disappearance and urea formation, and to attach considerable weight to the possible formation of nitrogenous intermediates of an undetermined sort.   So it is that we prefer to explain our findings with dl-alanine by concluding that part of the amino nitrogen has given rise to urea and part to undetermined substances in which the nitrogen behaves neither as primary amino nitrogen nor as urea. The fact that urea formation ceased at the 55 per cent level is probably fortuitous and does not, in our judgment, permit the inference that only one isomer gives rise to urea.

This interpretation is perhaps supported by observing that d-glutamic acid, the naturally occurring form, gives amino nitrogen and urea curves strikingly similar to those obtained with dl-alanine. Here the question of differences in the utilization of d and l forms is clearly not involved although the same premature cessation in urea formation is observed.

After oral administration, deamination of glutamic acid proceeded much more slowly, probably because of the slow absorption of glutamic acid from the alimentary canal (12, 13).   Unfortunately we have no record of the rate of urea formation.

It should be pointed out by way of contrast that alanine in our experience has never appeared toxic to rats. Glutamic acid,

however, invariably causes distress, as indicated by restlessness and profound polyuria. In both dogs and rats the areas exposed to subcutaneous injections of glutamic acid become edematous for several hours and (in dogs) frequently develop into open necrotic sores which heal very slowly (cf. also (14, 15)).

Glycine and aspartic acid behaved in a fashion quite unlike alanine and glutamic acid. Not only was deamination far from complete, even after 12 hours, but to our surprise the formation of urea was much in excess of the quantity expected and was still in rapid progress at the end of the experiment. An initial lag in urea formation, 6 to 8 hours in duration, was first observed, followed by a second phase in which the urea formed exceeded the nitrogen rendered available by deamination.

Orally administered glycine suffered a somewhat different fate. Deamination proceeded, in the later period, more rapidly and completely, while from the very beginning urea formation kept pace with deamination. There was no lag in its formation; indeed, an actual excess of urea seems indicated. We suspect that this difference in the metabolism of orally and subcutaneously administered glycine is due to differences in the rate of absorption and in the opportunities presented for prompt localization and deamination in the liver.

With aspartic acid an additional phenomenon of some interest was observed. After 3 or 4 hours, in which time 36 per cent of the amino nitrogen disappeared, further deamination seemed to be interrupted. Indeed, the amino nitrogen content of the animal actually increased until after 12 hours but 25 per cent of the injected amino nitrogen would seem to have disappeared. In view of the marked and uninterrupted formation of urea we are led to the conclusion that aspartic acid stimulated both the endogenous formation of urea and hydrolysis of tissue protein.

It is possible that the failure of glycine to undergo complete deamination, the process apparently coming to an end after 58 per cent was deaminized, is due to a similar phenomenon. If glycine were to stimulate tissue protein hydrolysis in like fashion, the decrease in amino nitrogen due to glycine deamination would be countered by an increase due to the hydrolytic liberation of amino acids. The deamination curve would therefore flatten out, or, as in the case of aspartic acid, actually pass through a point of inflection.

It is evident, of course, that a similar premature flattening of the deamination curve would be observed if any appreciable portion of the amino acid were to be excreted.   The loss of 40 per cent of an amino acid by excretion would necessarily cause the curve of amino nitrogen decrease to flatten out at the 60 per cent level. We have, however, given proportionately large doses of glycine to dogs without obtaining any significant increase in urinary amino nitrogen, and, in consequence, are unwilling to attach any weight to this possibility.

Tyrosine, administered subcutaneously in aqueous suspension, was also studied.   Even after 12 hours there was little if any absorption as indicated by maintenance of the amino nitrogen content at the high postinjection level.   In consequence, the urea values were but little greater than those of the controls.

Two main conclusions have come to us in the consideration of these findings. The unexpected dissociation between urea formation and amino nitrogen decrease seems to us to be the most striking. The primary lag in urea formation suggests that calculations of the extent of amino acid metabolism from the amount of urea excreted during the first few hours following amino acid administration are likely to be seriously incorrect.

This source of error has not escaped recognition by other investigators. Wilhelmj and Bollman (7) have already referred to the delayed elimination of urea in short period experiments as an assumption which seems to be fully warranted by the relatively small amount of urea excreted, which, in experiments lasting from 3.41 to 4.35 hours, was sufficient to account for 30 to 49 per cent of the amino acid given. In two other experiments extending over 6.88 and 8.72 hours, about 70 per cent of the theoretical amount of urea was excreted. Wilhelmj and Mann (16) observed that 55 to 92 per cent of the glycine or alanine administered to dogs fasted 6 to 14 days could not be accounted for. Their experiments were 4 hours in duration. Likewise Ralli, Canzanelli, and Rapport (17) recognized a pronounced lag in urea excretion during short period experiments as an accepted fact in making the calculations presented in their recent paper.

In experiments extending over 4 to 5 hours, Lusk (18) was able to account for only 20 to 45 per cent of the nitrogen administered to dogs in the form of glycine, alanine, glutamic acid, and leucine.

The urinary urea and ammonia increases indicated that only 14 to 32 per cent had been metabolized. On the basis of sugar formation in the phlorhizinized animal, it would seem, however, that 80 or 90 per cent of glycine and alanine must have undergone metabolism in a 5 hour period (19). Such an anomaly can only be explained by assuming that amino acid catabolism proceeds much more rapidly in the phlorhizinized animal, or else by recognizing that urea formation does not give an adequate picture of the rate of amino acid catabolism in short period experiments. In the lack of evidence bearing on the first of these possibilities, Lusk (20) accepted the second and chose to calculate the degree of glycine and alanine catabolism by use of Csonka's results on the rate of sugar excretion.

We wish to suggest that this well known lag in the *excretion* of urea may more properly be regarded, under certain conditions, as a lag in the *formation* of urea. It is evident, for example, that the more rapid excretion of sugar described by Reilly, Nolan, and Lusk (21), Csonka (19), and Janney (22), when proteins or amino acids (19) are fed to phlorhizinized dogs, may indeed indicate a true delay in the formation of urea. This fact is frequently interpreted to mean that the kidney of an animal under phlorhizin is more permeable to glucose than to urea, although Janney (22) pointed out the alternative explanation which we have again mentioned here. It should also be remarked that certain of the facts observed by Van Slyke and Meyer, and Folin and his associates in their well known studies of amino acid metabolism are pertinent to our own investigation. Thus Van Slyke and Meyer (23), referring to the decrease in hepatic amino nitrogen from the high level reached in the early postinjection period, state that "only a fraction of the amino nitrogen which disappears from the liver reappears as urea in the blood." The authors suggest, however, that the nitrogen might be more fully accounted for if estimates of both blood and tissue urea were available. Finally, Folin and Denis (24) and Folin and Berglund (25), concerned with the same phenomenon, showed that urea formation proceeds but slowly in the early stages of amino acid metabolism even though the amino nitrogen content of both blood and liver may be already on the wane.

The existence of nitrogen in some undetermined form following

amino acid administration has, furthermore, been recognized by direct blood analysis. Thus, Johnston and Lewis (15) obtained pronounced increases in the residual nitrogen of the blood, of such a magnitude that they could not be regarded as due to a mere summation of errors in the urea, amino nitrogen, and non-protein nitrogen determinations. The greatest increases were obtained with glycine. The effect was transient in character, but persisted over 6 to 12 hours. In our own experiments the presence of undetermined nitrogen following the subcutaneous administration of glycine was correspondingly transitory and lasted about 8 hours. Coincident with the increase in undetermined nitrogen of the blood, Johnston and Lewis observed that analyses of the urine fractions "frequently indicated a depression or at least no increase in urea excretion in the 6 hours immediately following the administration of glycine."   Silberstein, Rappaport, and Wachstein (26) observed similar transitory increases in the undetermined nitrogen of blood after the administration of peptone and proteins to dogs.   There was no increase in the polypeptide nitrogen of the trichloroacetic acid filtrate.   This would seem to discredit the possibility of peptide formation, which even from a priori considerations would seem improbable after administration of a single amino acid.   Van Slyke and Meyer (23) had occasion to comment on the possibility of injected glycine giving rise to body protein and agreed that such was unlikely.   In support of this belief they found no increase in the peptide nitrogen of the liver.

We have given some thought to the possibility of ammonia being a major constituent of this undetermined fraction but preliminary experiments designed to inquire into the question have not given it support.   The work of Bornstein and Roese (27, 28) demonstrates quite clearly that the "flooding" of an animal with amino acids, glycine particularly, is productive of relatively great increases in the ammonia content of the blood.   At the same time, it should be recognized that the increases are quite insufficient to account for the undetermined nitrogen in our experiments, unless it be that increases in blood ammonia are accompanied by much greater increases in tissue ammonia.

A second conclusion that we have ventured to draw from these observations is that amino acids under certain conditions may

actually stimulate endogenous protein metabolism in a measure that has hitherto been seldom recognized. This fact seems clear from the existence of a second phase (glycine and aspartic acid) in which the urea formed exceeds the quantity obtainable from the amino nitrogen that has disappeared. One of us (29) has already reported that the excretion of sulfate by dogs following the subcutaneous injection of amino acids is so increased as to lend support to our concept of stimulation of metabolism.

The only related investigation in which the method of subcutaneous injection has been used is that of Thompson (30) in which arginine was given to dogs. Pronounced increases in endogenous protein metabolism (excess excretion of urea) were observed in experiments of long duration (24 hours). Occasionally, however, other investigators have found that amino acids in long period experiments stimulate nitrogen metabolism. It is apparent in the results of Abderhalden and Teruuchi (31) with glycine and glycylglycine (*per os*), in those of Krzywanek (32) with alanine (*per os* and intravenously), and in Stolte's experiments (33) in which a relatively large amount of glycine was given intravenously.

At the same time it is perfectly apparent that this increase in protein metabolism observed in long period experiments is dependent upon the conditions of the experiment, such as the duration of the experiment, the amount of amino acid given, the mode of administration, and the species employed. Thompson (30) observed no stimulation of metabolism when arginine was given to dogs *per os*, nor did Abderhalden and Teruuchi (31) when glycylglycine and alanylalanine were given subcutaneously. Schultzen and Nencki (34) did not obtain more than the calculated amount of urea when glycine and leucine were given to dogs (*per os*). In the same sense the results of von Knierem (35) (aspartic acid and asparagine, *per os*), Salkowski (36) (glycine and alanine, *per os*), and Levene and Meyer (6) (alanine, leucine, aspartic acid, and arginine, *per os*) may be considered negative.

We would also emphasize the fact, apparent in our own studies, that the primary lag in urea formation and subsequent stimulation of metabolism by glycine and aspartic acid may be peculiar to amino acids subcutaneously injected. It is significant that glycine, *per os*, was metabolized without any lag in urea formation or without

any late stimulation of protein metabolism. The formation of urea would seem to be persistently greater than amino nitrogen disappearance but we are not certain that the gap between the two curves is great enough to be significant.

The results presented in this paper are of some importance in the consideration of such a phenomenon as the specific dynamic action of amino acids and we have been interested in attempting an explanation of some of its obscurities with the aid of these additional facts. We are satisfied, however, that still more information is needed before the desired correlation can be made. Above all, much more must be learned about the fate of individual amino acids in the liver and muscles after subcutaneous and oral administration, and further inquiries must be made into the energy changes involved. This is now in progress.

SUMMARY

1. The decrease in amino acid nitrogen and the increase in urea which proceed in animals pursuant to the injection of amino acids were studied in fasted rats by analysis of the entire carcass and excreta at appropriate intervals after injection.

2. Although the amino nitrogen content of animals injected with *dl*-alanine fell rapidly from the high postinjection level to the base-line, little more than 50 per cent of the nitrogen was converted into urea.

3. An initial lag in urea formation, extending over 6 to 8 hours, was observed in the subcutaneous administration of glycine and *l*-aspartic acid. A second phase, in which the amount of urea formed exceeded that which could be obtained from the nitrogen of deamination, was also observed.

4. *l*-Aspartic acid was found to stimulate hydrolysis of tissue protein.

5. Glycine administered *per os* was metabolized without any lag in urea formation and with little, if any, stimulation of endogenous protein metabolism.

6. Under the conditions of these experiments, the formation of nitrogenous intermediates is postulated. The nitrogen of these undetermined substances does not react with nitrous acid but, ultimately, is converted into urea.

BIBLIOGRAPHY

1. Kiech, V. C., Luck, J. M., and Smith, A. E., *J. Biol. Chem.*, 90, 677 (1931).
2. Kiech, V. C., and Luck, J. M., *J. Biol. Chem.*, 77, 723 (1928).
3. Allen, F. W., and Luck, J. M., *J. Biol. Chem.*, 82, 693 (1929).
4. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 70, 557 (1926).
5. Abderhalden, E., and Schittenhelm, A., *Z. physiol. Chem.*, 51, 323 (1907).
6. Levene, P. A., and Meyer, G. M., *Am. J. Physiol.*, 25, 214 (1909).
7. Wilhelmj, C. M., and Bollman, J. L., *J. Biol. Chem.*, 77, 127 (1928).
8. Ringer, A. J., and Lusk, G., *Z. physiol. Chem.*, 66, 106 (1910).
9. Wohlgemuth, J., *Ber. chem. Ges.*, 38, 2064 (1905); *Biochem. Z.*, 1, 332 (1906).
10. Abderhalden, E., and London, E. S., *Arch. ges. Physiol.*, 212, 735 (1926).
11. Abderhalden, E., and Franke, K., *Fermentforschung*, 10, 39 (1929).
12. Seth, T. N., and Luck, J. M., *Biochem. J.*, 19, 366 (1925).
13. Wilson, R. H., and Lewis, H. B., *J. Biol. Chem.*, 84, 511 (1929).
14. Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 36, 9 (1918).
15. Johnston, M. W., and Lewis, H. B., *J. Biol. Chem.*, 78, 67 (1928).
16. Wilhelmj, C. M., and Mann, F. C., *Am. J. Physiol.*, 93, 69 (1930).
17. Ralli, E. P., Canzanelli, A., and Rapport, D., *Am. J. Physiol.*, 96, 331 (1931).
18. Lusk, G., *J. Biol. Chem.*, 13, 155 (1912–13).
19. Csonka, F. A., *J. Biol. Chem.*, 20, 539 (1915).
20. Lusk, G., *J. Biol. Chem.*, 20, 555 (1915).
21. Reilly, F. H., Nolan, F. W., and Lusk, G., *Am. J. Physiol.*, 1, 395 (1898).
22. Janney, N. W., *J. Biol. Chem.*, 20, 321 (1915).
23. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 16, 213 (1913–14).
24. Folin, O., and Denis, W., *J. Biol. Chem.*, 11, 87 (1912).
25. Folin, O., and Berglund, H., *J. Biol. Chem.*, 51, 395 (1922).
26. Silberstein, F., Rappaport, F., and Wachstein, M., *Biochem. Z.*, 213, 312 (1929).
27. Bornstein, A., and Roese, H. F., *Biochem. Z.*, 212, 127 (1929).
28. Bornstein, A., *Biochem. Z.*, 212, 137 (1929).
29. Luck, J. M., and Amsden, M. R., *J. Biol. Chem.*, 92, p. lxv (1931).
30. Thompson, W. H., *J. Physiol.*, 32, 137 (1905); 33, 106 (1906).
31. Abderhalden, E., and Teruuchi, Y., *Z. physiol. Chem.*, 47, 159 (1906).
32. Krzywanek, F. W., *Biochem. Z.*, 134, 500 (1922).
33. Stolte, K., *Beitr. chem. Physiol. u. Path.*, 5, 15 (1904).
34. Schultzen, O., and Nencki, M., *Ber. chem. Ges.*, 2, 566 (1869); *Z. Biol.*, 8, 124 (1872).
35. von Knierem, W., *Z. Biol.*, 10, 263 (1874).
36. Salkowski, E., *Z. physiol. Chem.*, 4, 100 (1880).

THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI

XXIV. ANALYSIS OF THE ACETONE-SOLUBLE FAT OF THE BOVINE TUBERCLE BACILLUS*

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INTRODUCTION

The composition of the fat fractions of the bovine tubercle bacillus was practically unknown previous to the work done in this laboratory. The available information concerning the lipoids of this important organism was summarized in Paper XII of this series (1) and the composition of the phosphatide fraction was described in Paper XIX (2).

The present report deals with the analysis of the acetone-soluble fat; we have followed the methods outlined in the study of the analogous fraction from the human bacillus (3). The acetone-soluble fat was found to consist largely of free fatty acids together with a small amount of neutral fat. The neutral fat is not a glyceride because glycerol could not be isolated after the fat had been saponified. Some substance other than glycerol, possibly a polyhydric alcohol, is contained in the aqueous solution after saponification, but this substance could not be identified.

The aqueous solution also contained a trace of a water-soluble volatile fatty acid. This acid had an odor similar to that of butyric acid but the amount of material obtained was too small to permit of a definite identification.

The solid saturated fatty acids consisted of palmitic acid and cerotic acid.   Stearic acid could not be identified.   The liquid fatty acids, after distillation of their methyl esters, were reduced with hydrogen and platinum oxide (4).   The reduction product was separated by means of the lead soap-ether procedure and it was found to consist of stearic acid.   We may conclude from the iodine number of the mixed acids and the amount of stearic acid obtained after reduction that the unsaturated acids contained in the fat must have consisted of linoleic acid and linolenic acid rather than of oleic acid.

The liquid saturated fatty acids obtained from the ether-soluble lead salts, after separation of the reduced acid, were esterified and the methyl esters were distilled in a high vacuum, yielding a large

TABLE I

Constants of Acetone-Soluble Fat

	Bovine tubercle bacillus	Human tubercle bacillus
Melting point, °C	38	33
Iodine No.	48.4	52.6
Acid value	117.5	60.3
Saponification value	171.2	203.6
Ester value	53.7	143.2
Reichert-Meissl value	4.15	3.96
Unsaponifiable matter	9.7	10.3

amount of a low boiling fraction and a small amount of a high boiling ester.   The low boiling ester yielded on saponification a liquid saturated fatty acid that was similar to tuberculostearic acid (5).   The amount of the high boiling ester was too small to permit of adequate purification but the results obtained indicate that a higher saturated fatty acid of low melting point is contained in this fraction.   This acid differs from phthioic acid (5) in that it is optically inactive.

The biological reactions of the acetone-soluble fat and of the liquid saturated fatty acids mentioned above are being studied in Dr. Sabin's laboratory at the Rockefeller Institute.   The results are not yet available but they will be reported separately by Dr. Sabin.

The acetone-soluble fat used in this investigation had been isolated by Anderson and Roberts as described in Paper XII of this series (1). It formed a reddish brown soft mass and possessed a distinctly agreeable odor. The fat contained at the most only minute traces of phosphorus, while nitrogen and sulfur were absent. The constants of the fat were determined according to the methods described by Grün (6) and the values found are given in Table I. The constants of the acetone-soluble fat of the human bacillus as determined by Anderson and Chargaff (3) are included for comparison.

The data in Table I indicate that the fat from the bovine bacillus contained about twice as much free fatty acids as the human bacillus and a correspondingly smaller amount of neutral fat. The fat contained a small amount of volatile fatty acids but the value given in Table I is believed to be too high.

Saponification of the Fat

Two portions of the fat, weighing 52 gm. each, were saponified by refluxing with 300 cc. of 4 per cent alcoholic potassium hydroxide in an atmosphere of nitrogen[1] for 5 hours. After about one-half of the alcohol had been distilled off, the soap solution was diluted with 2 liters of water and extracted seven times with 500 cc. of ether. The ethereal extract was washed with water, dried with sodium sulfate, filtered, and the ether was distilled. The unsaponifiable matter thus obtained formed a dark brown oil and after drying in a vacuum desiccator it weighed 13.7 gm. The iodine number was 109 but the substance gave no definite sterol color reactions. Lack of time has prevented any further examination of this fraction which is reserved for a future study.

Water-Soluble Constituents

The soap solution was acidified with hydrochloric acid and the fatty acids were extracted with ether. The ethereal solution was

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[1] During all operations until the liquid acids were reduced, the fatty acids were protected from air by an atmosphere of carbon dioxide or nitrogen. All solvents used in this work had been freshly distilled and the alcohol had been distilled over potassium hydroxide.

washed with water and the fatty acids were isolated, as will be described later.   The aqueous solution and washings were evaporated under reduced pressure to a volume of about 200 cc.   The distillate was saved and examined for water-soluble volatile fatty acids.   The concentrated aqueous solution was filtered to remove the potassium chloride that had separated and the filtrate was evaporated to dryness *in vacuo*.   The residue was evaporated several times after additions of absolute alcohol to remove most of the water and it was then extracted with absolute alcohol. The alcoholic extract was evaporated to dryness and the residue was again extracted with absolute alcohol, filtered, and then evaporated to dryness *in vacuo*.   The residue was again taken up in absolute alcohol, the solution was filtered, and the solvent was evaporated *in vacuo*.   The syrupy residue which remained, after it had been dried in a vacuum desiccator, weighed 9.7 gm., corresponding to 9.2 per cent of the fat.   When some of the substance was heated with acid potassium sulfate, an acrid odor similar to that of acrolein was noticed.   An unsuccessful attempt was made to isolate any glycerol that might be present in the residue by distillation at a pressure of about 20 mm.   When the temperature of the bath reached 150°, noticeable decomposition was taking place and distillation was interrupted.   About 0.6 gm. of a light yellowish oily distillate had gone over, but this substance was not glycerol.   It solidified on cooling in ice water and was insoluble in water.   It was readily soluble in alcohol but insoluble in ether. The dark colored residue in the distillation flask was extracted with hot alcohol and the extract was filtered, leaving an insoluble dark powder that weighed 0.7 gm.   The alcoholic solution was evaporated to dryness, when 4 gm. of a thick syrupy substance remained.   These substances have not been examined any further, but the results indicate that very little, if any, glycerol could have been present.

Volatile Fatty Acids

The aqueous distillate mentioned above was again distilled over a free flame.   The first quarter was collected and after it had been neutralized with potassium hydroxide, the solution was evaporated to dryness.   The residue was dissolved in 500 cc. of water, acidified with phosphoric acid, and distilled over a free flame until the distillate was neutral.   The volatile fatty acids contained in

the distillate were neutralized with potassium hydroxide, and the solution was evaporated to dryness, when 0.2 gm. of a potassium salt was obtained. When some of this substance was acidified with dilute sulfuric acid, a sharp unpleasant acid odor was noticeable, similar to that of butyric acid. The amount of volatile acids obtained was so small that a definite identification was impossible.

Isolation and Separation of Fatty Acids

The ethereal solution of the fatty acids was dried with sodium sulfate, filtered, washed with ether, and evaporated to dryness. The crude fatty acids formed a brownish red soft mass which, after drying in a vacuum desiccator, weighed 87.9 gm. The material was dissolved in 200 cc. of alcohol, neutralized with potassium hydroxide, diluted with an equal volume of water, and the solution was poured into an excess of 20 per cent aqueous lead acetate solution. The lead soaps were filtered off, washed with water, and after drying in a vacuum desiccator they were treated with 500 cc. of ether. The ether-insoluble and the ether-soluble lead soaps were decomposed in the usual manner with dilute hydrochloric acid, yielding 33.8 gm. of solid acids and 47.6 gm. of liquid acids.

Solid Fatty Acids

The solid fatty acids were dissolved in methyl alcohol, decolorized with norit, and twice recrystallized from methyl alcohol. The white crystalline substance melted at 54–55°, and the molecular weight, determined by titration, was 277. Further recrystallizations did not cause any change in the melting point, and it was apparently impossible to separate the fatty acids by crystallization. The acids were therefore converted into the methyl ester and the latter was fractionated in a vacuum. The following fractions were collected.

Fraction No.	Temperature of bath	Boiling point of ester	Weight	Pressure
	°C.	°C.	gm.	mm.
1-a	125–135	112–120	24.1	0.005
2-a	135–155	117–126	3.5	0.003
3-a	155–180	126–143	1.0	0.003
4-a	Residue in flask		2.8	

Fraction 1-a was again distilled at a pressure of about 0.002 mm., when 23 gm. of the ester, Fraction 1-b, went over at a temperature of 101°.

The residue in the flask was combined with Fraction 2-a and the mixture was distilled. The first run was rejected but the main portion, Fraction 1-c, 4.6 gm., went over at between 120–130° at a pressure of about 0.005 mm.

Isolation of Palmitic Acid

Fraction 1-b melted at 28–28.5°. The following values were obtained on analysis.

2.728 mg. substance: 3.06 mg. H_2O and 7.54 mg. CO_2.
2.505 "   "   : 2.79 "   "   " 6.95 "   "
Calculated for $C_{15}H_{31}O_2CH_3$ (270). C 75.55, H 12.59
Found. " 75.38, " 12.56
" 75.66, " 12.47

It is evident from the data given above that Fraction 1-b, which constitutes the greater portion of the solid acids, is practically pure methyl palmitate.

The ester was saponified with alcoholic potassium hydroxide and the free acid isolated in the usual manner. It formed a white crystalline solid and weighed 21.6 gm. After two crystallizations from methyl alcohol the acid melted at 63–64°, and two further crystallizations from acetone caused no change in the melting point. A mixed melting point with pure palmitic acid showed no depression. The following analytical values were obtained.

3.030 mg. substance: 3.44 mg. H_2O and 8.385 mg. CO_2.
2.789 "   "   : 3.155 "   "   " 7.725 "   "
Calculated for $C_{16}H_{32}O_2$ (256). C 75.00, H 12.50
Found. " 75.47, " 12.70
" 75.54, " 12.65

Titration—1.2235 gm. of acid dissolved in neutral alcohol, with phenolphthalein as indicator, required 47.0 cc. of 0.1 N KOH.

Calculated for $C_{16}H_{32}O_2$. Mol. wt. 256
Found. "   " 260

Isolation of Cerotic Acid

Fraction 4-a, which remained as a residue in the first distillation, was saponified with alcoholic potassium hydroxide, and the free acid was isolated, yielding 2.7 gm. of a slightly yellowish crystalline solid. After two crystallizations from methyl alcohol the substance melted at 80–82°, and two further crystallizations from acetone raised the melting point to 84–85°. The substance was again recrystallized three times from acetone but there was no change in the melting point. The acid crystallized from acetone in beautiful snow-white plates; the final yield was 2.2 gm. The analysis gave the following results.

2.802 mg. substance: 3.28 mg. H_2O and 8.08 mg. CO_2.
2.779 "   "   : 3.26 "   "   " 8.015 "   "
          Calculated for $C_{26}H_{52}O_2$ (396). C 78.78, H 13.13
                    Found. " 78.64, " 13.09
                           " 78.65, " 13.12

Titration—0.5094 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 12.91 cc. of 0.1 N KOH.

          Calculated for $C_{26}H_{52}O_2$. Mol. wt. 396
                    Found. "   " 394

The silver salt was prepared as follows: 0.38 gm. of the acid was dissolved in 25 cc. of absolute alcohol, neutralized with 0.1 N alcoholic KOH, and diluted with 25 cc. of water. A slight excess of silver nitrate dissolved in 50 per cent alcohol was added, when a white precipitate separated. The silver salt was filtered off, washed with dilute alcohol, and dried to constant weight *in vacuo*. For analysis the substance was burned at a low heat in a porcelain crucible and the residue of metallic silver was weighed.

0.1993 gm. substance: 0.0437 gm. Ag.
          Calculated for $C_{26}H_{51}O_2Ag$ (502.88). Ag 21.45
                    Found. " 21.92

The properties of the high melting acid are identical with those found by Anderson and Chargaff (3) for the cerotic acid isolated from the acetone-soluble fat from the human tubercle bacillus, and the analytical values agree closely with the calculated composition of hexacosanic acid. The amount of the acid obtained,

2.7 gm., is equal to 2.6 per cent of the fat; this value is minimal since it is impossible by present methods to separate such a substance quantitatively.

Examination of Fraction 1-c

The quantity of Fraction 1-c was too small to permit of an adequate purification by fractional distillation. The fraction was therefore saponified, and the free acid was isolated and recrystallized twice from methyl alcohol. The yield was 3 gm. of snow-white crystals that melted at 65–67°; the molecular weight, determined by titration, was 293. The high melting point and the high value for the molecular weight would indicate that this intermediate fraction consisted of a mixture of palmitic acid and hexacosanic acid rather than of palmitic and stearic acids.

We believe therefore that the solid saturated fatty acids consisted mainly of palmitic acid together with a smaller amount of hexacosanic acid.

Examination of Liquid Fatty Acids

The liquid fatty acids obtained from the ether-soluble lead soaps were obtained as a dark colored oil which weighed 47.6 gm., representing therefore 45 per cent of the fat and 54 per cent of the total fatty acids. The iodine number, determined by the Hanus method, was 65.6. The fatty acids could not be decolorized with norit and it was found to be impossible to reduce the unsaturated acids with hydrogen and platinum oxide because some impurity was apparently present which poisoned the catalyst.

The crude liquid acids were converted into methyl esters, and the ethereal solution of the esters was extracted first with dilute sodium carbonate and then with 2 per cent sodium hydroxide. The alkaline extraction removed 1.0 gm. of a dark colored oily substance which was not further examined. The ethereal solution, after it had been dried with sodium sulfate, was concentrated and the residue was dried *in vacuo*, leaving 42 gm. of a dark colored oil. The esters were distilled in a high vacuum, when 33 gm. of a light yellow oil went over below 200° and 9 gm. of a nearly solid material remained in the distillation flask.

Catalytic Reduction of Methyl Esters

The distilled methyl esters, 33 gm., were dissolved in ethyl acetate and reduced with hydrogen and platinum oxide. The platinum black was filtered off and the solvent was removed by distillation. The residue was saponified with alcoholic potassium hydroxide, and the free acids were isolated and separated into solid and liquid acids by repeating the lead soap-ether treatment.

Reduced Fatty Acid

The solid fatty acid obtained from the ether-insoluble lead soaps was a white crystalline solid which weighed 11 gm., equivalent to 10.5 per cent of the fat and 12.5 per cent of the total fatty acids. The substance was crystallized twice from methyl alcohol and twice from acetone, yielding 5.5 gm. of snow-white crystals. The acid melted at 70–71° and there was no change in the melting point after two further crystallizations from acetone. A mixed melting point with pure stearic acid showed no depression. On analysis the following values were found.

2.530 mg. substance: 2.88 mg. H_2O and 7.095 mg. CO_2.
2.940 "   "   : 3.425 "   "   " 8.255 "   "
Calculated for $C_{18}H_{36}O_2$ (284). C 76.05, H 12.67
Found. " 76.48, " 12.73
" 76.57, " 13.03

Titration—1.1640 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 40.3 cc. of 0.1 N KOH.

Calculated for $C_{18}H_{36}O_2$. Mol. wt. 284
Found. "   " 288

The mother liquors from the crystallizations mentioned above were concentrated, and the acid which separated was crystallized three times from acetone. The snow-white crystals melted at 69–70°. The final mother liquors on concentration yielded about 1 gm. of an acid fraction which melted at 57–58°. It is evident, however, from the data given above that the reduced acid consisted of nearly pure stearic acid.

The comparatively high iodine number, 65.6, of the liquid fatty acids and the small amount of stearic acid obtained by reduction indicate that the bulk of the unsaturated acids must have consisted of linoleic acid or linolenic acid rather than of oleic acid.

Saturated Liquid Fatty Acids

The liquid fatty acids, isolated from the ether-soluble lead soaps after the catalytic reduction, were dissolved in alcohol and treated a second time with hydrogen and platinum oxide, but no hydrogen was absorbed.   After the platinum black was filtered off, the solvent was removed by distillation and the oily residue was dissolved in ether.   The solution was dried with sodium sulfate, filtered, washed with ether, and the ether was distilled.   The liquid fatty acids formed a slightly yellowish oil which weighed 19 gm.   The substance was saturated, since no iodine was absorbed when tested by the Hanus method.   An alcoholic solution of the acids was optically inactive, and the molecular weight, determined by titration, was 309.6.

The liquid saturated fatty acids were esterified by refluxing with absolute methyl alcohol containing dry hydrochloric acid.   About one-half of the alcohol was removed by distillation and the reaction mixture was diluted with water.   The esters were extracted with ether and the ethereal extract was washed with 5 per cent sodium carbonate, 2 per cent sodium hydroxide, and with water. The ethereal solution, after it had been dried with sodium sulfate, filtered, and washed with ether, was evaporated to dryness.   The oily residue, after it had been dried in a vacuum desiccator, weighed 17.3 gm.

The esters were fractionated in a high vacuum, yielding two principal fractions.   Fraction I distilled mostly at 130°, at 0.004 mm. pressure, as a slightly yellowish mobile oil.   It weighed 15.3 gm.   Fraction II was a thick yellowish oil, with a boiling point of about 170° at 0.003 mm. pressure, and weighed 1.5 gm.

Fraction I was saponified with alcoholic potassium hydroxide and the free acid was isolated and dried.   It was a slightly yellowish oil which weighed 14.3 gm.   On cooling in ice water, the acid solidified to a white crystalline mass and melted at 9–10°.   The acid was optically inactive.   The following values were obtained on analysis.

3.126 mg. substance: 3.495 mg. H_2O and 8.75 mg. CO_2.
3.745 "   "   : 4.27 "   "   "   10.475 "   "
Calculated for $C_{18}H_{36}O_2$ (284). C 76.05, H 12.67
Found.   " 76.34, " 12.51
" 76.28, " 12.75

Titration—0.5007 gm. and 0.5123 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 17.3 cc. and 17.6 cc. of 0.1 N KOH.

<div style="text-align:center">

Calculated for $C_{18}H_{36}O_2$. Mol. wt. 284
Found.   "   " 289, 290

</div>

The composition of this acid is the same as that of stearic acid, and the properties agree with those described previously for the tuberculostearic acid which was isolated from the acetone-soluble fat of the human tubercle bacillus.

Fraction II was saponified and the free acid was isolated in the usual manner. The acid was a thick, slightly yellowish, oil and weighed 1.3 gm. After the substance had been kept for some time in a sealed tube, it partly solidified, but it melted when the tube was warmed to 20–21°. In alcoholic solution the acid showed no optical rotation.

<div style="text-align:center">

6.357 mg. substance: 7.06 mg. H_2O and 18.06 mg. CO_2.
Found. C 77.52, H 12.43

</div>

Titration—0.5267 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 16.0 cc. of 0.1 N KOH.

<div style="text-align:center">

Found. Mol. wt. 329

</div>

The analytical values and the molecular weight agree approximately with an acid of the formula $C_{21}H_{42}O_2$. We do not believe that this substance is homogeneous but that it is a mixture; it will be necessary to secure larger quantities before this acid can be purified properly. The results indicate, nevertheless, that the fat contains at least two higher liquid saturated fatty acids which in a certain sense are analogous to the liquid saturated fatty acids that occur in the acetone-soluble fat of the human tubercle bacillus.

Non-Volatile Portion of Methyl Esters of Liquid Fatty Acids

The non-volatile portion of the crude methyl esters was saponified and an effort was made to separate the material into different fractions. The substances that were isolated were obviously mixtures of fatty acids of high molecular weight and low melting points from which no pure substances could be isolated. All of these fractions were optically inactive.

The composition of the acetone-soluble fat is given in Table II and the values obtained for the analogous fraction from the human bacillus are included for comparison.

We are indebted to Professor H. T. Clarke of Columbia University, in whose laboratory the micro analyses were made.

TABLE II

Composition of Acetone-Soluble Fat

	Bovine bacillus *dite* Vallée			Human bacillus, Strain H-37		
	Fat	Fatty acids	Iodine No.	Fat	Fatty acids	Iodine No.
	per cent	*per cent*		*per cent*	*per cent*	
Water-soluble..................	9.28			6.61		
Total fatty acids...............	83.90			83.33		
Unsaponifiable matter..........	13.10*		109	11.94		85.9
Solid fatty acids...............	32.40	38.50		30.38	36.45	
Liquid " "	45.00	54.00	65.66	50.65	60.78	53.8
Unsaturated fatty acids after reduction to stearic acid.......	10.60	12.50		12.59	15.11	
Liquid saturated fatty acids.....	18.60	21.70		38.06	45.67	

* This value is higher than that given in Table I because the preparation had only been dried in a vacuum desiccator.

SUMMARY

1. The acetone-soluble fats of the bovine and human tubercle bacilli are similar in composition.

2. The fat contains a large proportion of free fatty acids.

3. The neutral fat is not a glyceride but apparently represents esters of fatty acids with some higher polyhydric alcohol.

4. The fat contains a trace of a water-soluble volatile acid with an odor similar to that of butyric acid.

5. The solid saturated fatty acids consist of palmitic acid, $C_{16}H_{32}O_2$, and cerotic acid, $C_{26}H_{52}O_2$.

6. The unsaturated fatty acids yield stearic acid on reduction and probably consist of linoleic acid and linolenic acid.

7. The fat contains at least two liquid saturated fatty acids. One is an isomer of stearic acid which is similar to or identical with tuberculostearic acid. The other could not be obtained in very

pure form but it is apparently an acid of higher molecular weight than stearic acid and it is optically inactive.

8. Certain higher fatty acids which yield ether-soluble lead soaps are also present but this fraction could not be purified.

BIBLIOGRAPHY

1. Anderson, R. J., and Roberts, E. G., *J. Biol. Chem.*, **85,** 529 (1929–30).
2. Anderson, R. J., and Roberts, E. G., *J. Biol. Chem.*, **89,** 599 (1930).
3. Anderson, R. J., and Chargaff, E., *J. Biol. Chem.*, **84,** 703 (1929).
4. Voorhees, V., and Adams, R., *J. Am. Chem. Soc.*, **44,** 1397 (1922).
5. Anderson, R. J., and Chargaff, E., *J. Biol. Chem.*, **85,** 77 (1929–30).
6. Grün, A., Analyse der Fette und Wachse, Berlin (1925).

THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI

XXV. THE COMPOSITION OF THE PHOSPHATIDE FRACTION OF THE TIMOTHY BACILLUS*

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INTRODUCTION

The extraction and separation of the lipoids of the timothy bacillus into fractions consisting of phosphatide, acetone-soluble fat, and wax were described in Paper XXIII of this series (1). The composition of these fractions is now under investigation and we wish to report at this time the results obtained on analyzing the phosphatide.

The timothy bacillus yielded much less lipoid than the pathogenic acid-fast bacilli; the amount of phosphatide obtained from 1600 cultures was only 18.7 gm. Since the quantity of this rare preparation was so limited and as some of the material was also needed for biological investigations, it was necessary to use rather small amounts of substance for the chemical analyses. For this reason the losses on a percentage basis during the various operations were large and we could only identify about 70 per cent of the cleavage products.

After the phosphatide had been hydrolyzed, it yielded about 60 per cent of ether-soluble constituents, mainly fatty acids together with a small amount of waxy substance, and some 40 per cent of water-soluble material.   The cleavage products were separated by methods as similar as possible to those used in this laboratory in the analyses of other bacterial phosphatides (2).

The fatty acids consisted of a mixture of saturated solid acid and a liquid acid fraction.   The former was found to be practically pure palmitic acid, while the latter contained some unsaturated acid and a liquid saturated fatty acid.   The liquid acid fraction was first subjected to catalytic reduction and then separated by means of the lead soap-ether procedure.   The amount of reduced acid was too small to permit of adequate purification but the melting point and molecular weight were about the same as are usually found for a eutectic mixture of palmitic and stearic acids.   The liquid saturated fatty acid, obtained from the ether-soluble lead salts, was similar in physical properties and composition to the liquid saturated fatty acids isolated from the phosphatides of the avian and bovine bacilli but it differed from the crude phthioic acid of the human phosphatide in that it was optically inactive (2).

The water-soluble constituents consisted of glycerophosphoric acid, inosite, mannose, and some other reducing sugar that could not be identified.   The phosphatide contained a trace of nitrogen but the amount was so small that no effort was made to isolate any nitrogen compound.

It is evident from the cleavage products that were isolated and identified that the chemical composition of the timothy phosphatide is very similar to that of the phosphatides which have been studied previously from the human, avian, and bovine tubercle bacilli (2).

In view of the interesting biological reactions that have been obtained with the lipoids of the tubercle bacilli (3) it seemed a matter of importance to study by similar methods the fractions which have been isolated from the timothy bacillus, a non-pathogenic organism.   The biological experiments have not been completed but preliminary results reported from Dr. Sabin's laboratory at the Rockefeller Institute indicate that the timothy phosphatide gives reactions which are closely similar to those obtained from the

pathogenic tubercle bacilli.   In a certain sense this result is not surprising since all of the bacillary phosphatides are very similar in chemical composition and in physical properties, but, on the other hand, these findings would appear to complicate the question of specificity rather than explain differences in virulence of the acid-fast bacteria.

<div align="center">EXPERIMENTAL</div>

The phosphatide had been isolated and purified as described in Paper XXIII (1).   It was a yellowish, non-hygroscopic powder that melted with decomposition at 190°.   It contained 2.80 per cent of phosphorus and 0.22 per cent of nitrogen.   When the substance was rubbed up with water, it formed an opalescent colloidal solution which became turbid on addition of a few drops of sulfuric acid, and on heating to boiling a heavy coagulum was formed.   After the mixture had been heated for 2 hours, the coagulum was partly liquefied; it solidified on cooling.   The solid matter was filtered off and the neutralized filtrate was boiled with Fehling's solution, when a heavy reduction occurred.   The aqueous suspension of the original phosphatide when boiled with Fehling's solution gave no reduction.   It is evident, therefore, that the phosphatide contained no free reducing sugar but that reducing sugars were liberated on acid hydrolysis.

Hydrolysis of the Phosphatide

Two separate hydrolyses, in which 4.8 and 5.6 gm. of phosphatide were used, were carried out by refluxing the material with 120 cc. of 5 per cent sulfuric acid for 8 hours in an atmosphere of carbon dioxide.[1]   The coagulum which separated at first was gradually disintegrated, forming an oily layer on the aqueous solution, and the hydrolysis was considered complete when all solid particles had disappeared.   The mixture, after it had cooled, was extracted with ether and the ethereal solution was washed with water, dried, and the ether evaporated.   The residue, consisting of crude fatty acids, amounted to about 60 per cent of the phosphatide.

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[1] Throughout this work the phosphatide and the fatty acids were protected from the air by carbon dioxide or nitrogen until the fatty acids had been reduced.   Freshly distilled solvents were used, and the alcohol had been distilled over potassium hydroxide.

Water-Soluble Constituents

The aqueous portion of the hydrolysate was freed from sulfuric acid quantitatively by barium hydroxide, and after removing the barium sulfate the solution was concentrated under reduced pressure and made up to a volume of 100 cc. An aliquot of the solution was used for the determination of reducing sugars by the Munson-Walker method (4). The amount of reducing sugar calculated as glucose was 12.1 and 12.8 per cent.

Separation of Barium Glycerophosphate

The remainder of the solution was neutralized with barium hydroxide and mixed with an equal volume of alcohol. The bulky amorphous precipitate was filtered off, washed with dilute alcohol, and dried. The filtrate was saved for the isolation of the sugars. The crude barium salt from the two hydrolyses weighed 2.113 gm. It was treated with water and the insoluble portion was filtered off and washed with water. This fraction, which weighed about 0.5 gm., appeared to consist of inorganic barium phosphate and was discarded. The aqueous solution was precipitated by adding an equal volume of alcohol, and after repeating these operations about 0.6 gm. of a white amorphous powder was obtained. For analysis the substance was dried at 105° *in vacuo* over dehydrite. The loss in weight was 5.07 per cent.

0.2205 gm. dried substance:0.1534 gm. $BaSO_4$ and 0.0725 gm. $Mg_2P_2O_7$.
Calculated for $C_3H_7O_6P \cdot Ba + 2H_2O$ (343.4).   P 9.02, Ba 40.01
                                    Found.   " 9.16, " 40.93

The values found on analysis do not agree very closely with the calculated composition of barium glycerophosphate but for some reason it is always difficult to obtain better results in analyzing amorphous barium glycerophosphates. It is not impossible, however, that some organic phosphoric acid other than glycerophosphoric acid is present.

Isolation of Mannose Phenylhydrazone

The first filtrate from the barium glycerophosphate was concentrated under reduced pressure until most of the alcohol had been removed. The barium in the solution was removed quanti-

tatively by sulfuric acid and the barium sulfate was filtered off and washed with water. The filtrate was concentrated under reduced pressure to a volume of 20 cc. and the solution was mixed with 1 gm. of freshly distilled phenylhydrazine dissolved in 2 cc. of alcohol. The hydrazone began to crystallize almost immediately and after the mixture had stood overnight the crystals were filtered off, and washed with water, alcohol, and ether. The hydrazone obtained from the two hydrolyses weighed 1.302 gm., which corresponds to somewhat more than 9 per cent of mannose in the phosphatide.

The hydrazone was recrystallized from 60 per cent alcohol, when 0.72 gm. of slightly straw-colored crystals was obtained. When the substance was heated rapidly, it melted with decomposition at 200° and there was no depression of the melting point of a mixture with pure mannose phenylhydrazone which was heated simultaneously.

Analysis
0.1431 gm. substance: 13.80 cc. N_2 at 25° and 761 mm.
Calculated for $C_{12}H_{18}O_5N_2$ (270).   N 10.37
Found.   " 10.7

Isolation of Inosite

The filtrate from the mannose phenylhydrazone was shaken occasionally for 24 hours with an excess of benzaldehyde and the precipitated hydrazone was filtered off and discarded. After the filtrate had been extracted eight times with ether, it was concentrated to a syrup. The syrup resulting from the first hydrolysis was allowed to stand in a vacuum desiccator until colorless crystals of inosite separated. The crystals, after they had been collected on a small Buchner funnel, washed free of adhering syrup with cold 50 per cent alcohol, and dried, weighed 0.096 gm. The syrup from the second hydrolysis, after it had been dried *in vacuo,* was triturated with alcohol until a white powder was obtained. The latter was dissolved in a little dilute acetic acid and alcohol was added until the solution turned cloudy. On standing, colorless inosite crystals separated. The crystals were filtered off, washed with alcohol, and dried. The yield of crude inosite in this case was 0.097 gm.

The two lots of crystals were combined and purified by recrystal-

lization from dilute acetic acid by adding alcohol yielding colorless prismatic crystals that weighed 0.147 gm. The substance gave the reaction of Scherer and melted at 225°. There was no depression of the melting point when the substance was mixed with pure inactive inosite prepared from phytin.

Examination of Residual Syrup

The filtrate and washings from the crude inosite crystals were combined and evaporated to dryness. The syrup was treated with alcohol, when an insoluble residue was obtained. This residue was dissolved in water and since it reduced Fehling's solution it was heated with phenylhydrazine hydrochloride and sodium acetate. Typical glucosazone crystals began to separate after 10 minutes. After the osazone had been recrystallized from dilute alcohol, it melted with decomposition at 203°. A sample mixed with pure glucosazone when heated simultaneously melted at the same temperature. The amount of glucosazone obtained was too small to account for more than a fraction of the reducing substance.

The alcohol-soluble portion yielded on evaporation of the alcohol a syrupy residue. This substance also reduced Fehling's solution and when heated with acid potassium sulfate an unmistakable odor of acrolein was noticed. It would seem therefore that the syrup contained a mixture of reducing sugar and glycerol.

Examination of Fatty Acids

The total crude fatty acids weighed 6.28 gm. The material was dissolved in alcohol and the solution was neutralized with potassium hydroxide. A small amount of insoluble wax-like material was removed by filtration and washed with alcohol. After this substance had been dried *in vacuo*, it weighed about 0.5 gm. The fatty acids were separated by means of the lead soap-ether procedure into solid and liquid acids.

Solid Acid

The solid fatty acid, 2.14 gm., was dissolved in alcohol, neutralized with potassium hydroxide, and a small amount of insoluble material was filtered off. The solution was diluted with water, acidified, and extracted with ether. The fatty acid which was

recovered on evaporating the ether was completely soluble in alcohol and after it had been twice recrystallized from methyl alcohol was obtained as snow-white crystals. It melted at 61–61.5°.

Titration—0.3152 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 12.24 cc. of 0.1 N KOH.

Calculated for $C_{16}H_{32}O_2$. Mol. wt. 256
Found.   "   " 257

A second fraction of the acid was recovered by concentrating the mother liquors which melted at 59–61°; the molecular weight, determined by titration, was 257.8. We conclude therefore that the solid acid was practically pure palmitic acid.

Liquid Fatty Acids

The liquid fatty acids obtained from the ether-soluble lead salts weighed 3.1 gm.; the iodine number, determined by the Hanus method (4), was 26.2. This fraction also contained a slight amount of waxy material, 0.12 gm., which was removed by treatment with alcohol in which it was insoluble. The alcoholic solution of the liquid acid was reduced with hydrogen and platinum oxide (5) after which the lead soap-ether treatment was repeated.

Reduced Acid

The reduced acid obtained from the ether-insoluble lead salt weighed 0.58 gm., a quantity too small to permit of adequate purification. After one crystallization from methyl alcohol the acid melted at 55–57° and the molecular weight, determined by titration, was 266. These values are similar to those usually given by a mixture of palmitic acid and stearic acid.

Liquid Saturated Fatty Acid

The liquid fatty acid obtained from the ether-soluble lead salts, after the reduced acid was removed, was a mobile yellowish oil which weighed 1.88 gm. It was a saturated acid because it absorbed neither bromine nor iodine. It solidified when cooled in ice water and melted at between 8–11°. An alcoholic solution of the acid was optically inactive. The molecular weight determined

by titration was 380 to 383.   On combustion the acid gave the following values: C, 76.65, 76.56; H, 12.52, 12.63.

It is evident from these results that the substance is a mixture in which an acid of rather high molecular weight predominates. The acid is similar to the corresponding fractions isolated from the avian and bovine tubercle bacilli (2) but all of these acids differ from the crude phthioic acid (6) obtained from the human phosphatide which possesses optical activity.

The approximate composition of the timothy phosphatide as found in these analyses is as follows:

per cent

Mixed fatty acids.. 60
Palmitic acid.. 20
Reduced acid, probably a mixture of palmitic acid and stearic
    acid... 5.6
Liquid saturated fatty acid.............................. 18.0
Waxy material... 6.0
Loss in the separation and purification of fatty acids....... 10 6
Water-soluble material.................................. 40.0
Glycerophosphoric acid.................................. 10.0
Mannose.. 9.0
Inosite... 2.0
Unidentified water-soluble material...................... 19.0

BIBLIOGRAPHY

1. Chargaff, E., Pangborn, M. C., and Anderson, R. J., *J. Biol. Chem.*, **90**, 45 (1931).
2. Anderson, R. J., *J. Biol. Chem.*, **74**, 537 (1927).   Anderson, R. J., and Roberts, E. G., *J. Biol. Chem.*, **85**, 519 (1929–30); **89**, 599 (1930).
3. Sabin, F. R., and Doan, C. A., *J. Exp. Med.*, **46**, 645 (1927). Sabin, F. R., Doan, C. A., and Forkner, C. E., *J. Exp. Med.*, **52**, suppl. 3 (1930).
4. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 2nd edition (1925).
5. Voorhees, V., and Adams, R., *J. Am. Chem. Soc.*, **44**, 1397 (1922).
6. Anderson, R. J., *J. Biol. Chem.*, **83**, 169 (1929).

THE CALCIUM SALTS OF BONE

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These experiments were undertaken with the object of adding to the knowledge of the chemical character of the calcium salts of bone. Such knowledge would throw light on the factors governing the precipitation of calcium salts from body fluids not only in normal bone formation but in such pathological deposits as calcified tissues, hardening of the arteries, and gall-stones.

There are very few analyses on *unaltered* bone tissue available in the literature. The common procedure in studying the inorganic composition of bone has been to subject it to a preliminary ashing to get rid of the organic material; such treatment undoubtedly alters the inorganic constituents to some extent. Ashing not only converts some organic compounds of phosphorus to phosphates and breaks down any other organic compounds in which mineral elements may be held, but it may also decompose complex combinations between inorganic salts normally present in untreated bone, or may volatilize some of the inorganic constituents. Our data show that the degree of heat necessary for obtaining a white ash certainly alters the normal composition by decomposing some carbonate with the liberation of carbon dioxide.

In order to secure relatively pure samples of the inorganic portion of bone in which its constituents have been little, if at all, altered by preliminary treatment, we used only the dense outer portion (cortex) of the bone, in which the inorganic elements are especially concentrated, and analyzed this material (1) untreated, (2) dried, (3) after extraction at low temperatures with the various common solvents, (4) after removal of the proteins by hydrolysis with NaOH or by pancreatic digestion, and (5) after ashing at such low temperatures as to cause little or no loss of carbonate. All of

these preparations were made from a uniform, composite sample of bone cortex from cattle bones, thus eliminating any variations in composition due to differences in age or feeding of the animals, or to taking samples from different bones or even from different parts of the same bone. A composite sample from the inner portion of the bone was analyzed for comparison. All the bone preparations—and in addition two commercial preparations of supposedly c. p. tribasic calcium phosphate, one of high grade dibasic calcium phosphate, and one of calcite ($CaCO_3$)—were analyzed for their content of calcium, inorganic phosphorus, and carbon dioxide (carbonate). In reporting these analyses (Table II), details of parallel determinations have been omitted, but the figures given are each the result of several determinations, which usually checked very closely.

Analytical Procedure

The composite sample of pure bone cortex was obtained as follows: Cattle bones were broken into medium sized pieces, which were ground in a bone mill; the fine pieces of the dense outer portion of the bone were then mechanically separated from the other material and were ground in an iron mortar until of a fineness to pass through a 100-mesh sieve.

Samples were accurately weighed out on a piece of lead foil and the exact weight of each sample obtained by difference after transfer to the volumetric flask or Van Slyke and Neill apparatus (9), so that there was no loss by transfer. All samples were taken when the solids were dried to constant weight, a necessary precaution as they take up moisture readily. The size of the samples for CO_2 determinations varied slightly according to the carbonate content of the solid, but was usually about 20 mg. Samples taken for calcium and phosphorus determinations were as close as possible to 10 mg., and a factor was used to compute the results to an exact 100 mg. basis.

The technique suggested by Shear and Kramer (7) for dissolving the samples for Ca and P analyses was followed except that, after digestion with acid solution, addition of trichloroacetic acid, and making up to 10 cc. volume, the solution was cleared by centrifuging instead of by filtering. Calcium was then determined in triplicate on 2 cc. aliquots by the Kramer-Tisdall method (3),

the pH of the solution being adjusted after addition of brom-cresol purple. With the two best checks, the titrations practically always agreed to within 0.01 cc. of 0.01 N KMnO$_4$ solution.

Phosphorus determinations were made by the colorimetric method of Fiske and Subbarow (1). Usually a 2 cc. aliquot of the original acid solution of bone was taken, diluted to 10 cc., and a second aliquot of 2 cc. taken for colorimetric estimation (in 10 cc. volume), making the final sample 0.04 of the original sample. At this high dilution the HCl and trichloroacetic acid, added to the original sample to dissolve the bone and precipitate protein, can be ignored and molybdate reagent of the same degree of acidity (Molybdate I) can be added to both unknown and standard. We have calculated that this gives an acidity of 0.52 N in the unknown and of 0.51 N in the standard. It is essential that the degree of acidity should be the same in both solutions if the colors developed are to be comparable. We have also highly purified the aminonaptholsulfonic acid used as a reagent and used only freshly made up solutions. With this technique duplicate 2 cc. samples of the diluted aliquot (0.2 dilution) practically always gave perfect checks, while taking duplicate 2 cc. aliquots of the original acid solution of the sample and carrying each through the whole procedure gave results which were in almost as close agreement. In both the phosphorus and calcium determinations, accuracy of the volumetric samples was insured by using Van Slyke pipettes, calibrated to deliver a definite volume between two marks and scrupulously cleaned so that they drained well.

The method used for carbonate determinations was that proposed by Shear and Kramer (7) for direct measurement of the CO$_2$ content of bone in the Van Slyke and Neill (9) manometric blood gas apparatus. No difficulty was experienced in introducing the finely powdered solid apparently quantitatively into the apparatus through a stop-cock of 1 mm. bore with a small volume of wash water, but it was not possible to get as exact checks with solids as one is led to expect from experience in determining the CO$_2$ content of homogeneous solutions by this method.

Experimental Results

The figures given below show the untreated composite sample of bone cortex, which was the basis for all the bone preparations

analyzed, to have consisted of roughly 65 per cent inorganic material and 10 per cent moisture. The remaining 25 per cent is chiefly the organic matrix of the bone, although it may include some inorganic constituents which would be lost in ashing. The very small amounts of alcohol-ether-soluble material indicate the bone cortex to be almost free from fats and lipoids; water-soluble

TABLE I

Effects of Heating on Carbonate Content of Bone

Bone cortex	CO_2 calculated as	
	CO_2 per 100 mg. sample	$CaCO_3$ per 100 mg. sample
	mg.	*mg.*
Untreated, not dried............................	3.88	8.83
" dried...............................	4.01	9.11
Extracted with H_2O, alcohol, and ether (at room temperature)................................	4.13	9.38
Extracted with H_2O (at higher temperature, Soxhlet).....................................	3.73	8.47
Ashed to white ash over Meeker burner.........	1.03	2.34
" " " " " Bunsen " below dull red heat with NH_4NO_3...................	3.76	8.56
Ashed to brownish ash over Bunsen burner below dull red heat.............................	4.40	10.00
Inorganic residue left after NaOH hydrolysis....	4.58	10.43
" " " " pancreatic digestion	4.46	10.15

	per cent
Ash (white ash over Meeker burner).......................	64.98
Moisture..	9.72
Alcohol-soluble material (Soxhlet extraction)..............	0.38
Ether-soluble " " "	0.58
Water-soluble " " "	5.85

material is also limited by the fact that most of the proteins in bone matrix, as well as the inorganic salts of bone, are only very slightly soluble in water. Bone cortex, therefore, represents a relatively concentrated preparation of the inorganic constituents of bone, which may be further purified by procedures for getting rid

of protein material, such as mild hydrolysis, digestion, or ashing at low temperatures.

In Table I is given the carbonate content of the various bone cortex preparations after being subjected to the various procedures for freeing the inorganic material from organic constituents (as outlined above). As the carbonates are probably the most readily decomposed of the inorganic components, they may be regarded as an index of whether the treatment received is likely to have materially altered the inorganic composition of the bone. It will be seen that drying, extraction at room temperature, ashing below dull red heat, NaOH hydrolysis, and pancreatic digestion leave the carbonates unaffected (allowing for the slightly higher concentration due to removal of moisture and organic matter). More drastic treatment such as extraction with water in a Soxhlet apparatus and ashing at low temperatures with ammonium nitrate results in a very slight lowering of the carbonate content. However, the degree of heat ordinarily used in *ashing causes a decided loss of carbon dioxide* through decomposition of carbonates. Unless extreme care is taken to keep the temperature low, the CO_2 content is reduced from about 4 per cent (in untreated bone) to 1 per cent in the ash. Heating in a muffle furnace did not reduce the carbonate content further. It is almost impossible to secure a white ash without decomposing some carbonate, and other changes in the inorganic constituents may also be brought about by ashing.

Analyses for the calcium, inorganic phosphorus, and carbonate content of all our bone preparations, in which the inorganic components had been practically unaffected by previous treatment, and of supposedly pure samples of different calcium salts, are presented in Table II. Results are stated in mg. per 100 mg. of sample (per cent basis) and also calculated on a molarity basis. The latter is undoubtedly preferable since it allows direct comparisons as to relative amounts of the different molecules present, which give the clue as to how they may be combined. We have also calculated our ratios for residual Ca:P and $Ca_3(PO_4)_2$:$CaCO_3$ on a molarity basis.[1]

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[1] In the earlier papers dealing with the composition of untreated bone, these ratios have been computed on a weight basis. On untreated bone, our analyses, calculated on a weight basis, yield the value 1.91 for the ratio

Bone cortex	Calcium		Phosphorus		Carbon dioxide		CaCO₃	Resid-ual Ca	Residual Ca/P	Ca₃(PO₄)₂		mM Ca₃(PO₄)₂ / mM CaCO₃
										Calculated from Ca	Calculated from P	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	mg. per 100 mg.	mM	mg. per 100 mg.	mM	mg. per 100 mg.	mM	mM	mM	mM	mM	mM	
Untreated, dried................	26.5	0.662	11.98	0.387	4.01	0.091	0.091	0.571	1.47	0.190	0.193	2.1
Alcohol-extracted..............	27.4	0.685	12.44	0.402	4.23	0.096	0.096	0.589	1.46	0.196	0.201	2.0
Ether-extracted...............	31.7	0.792	12.13	0.392	4.33	0.098	0.098	0.693	1.76	0.231	0.196	2.3
Water-extracted (Soxhlet)......	28.3	0.708	12.21	0.394	3.73	0.085	0.085	0.623	1.58	0.208	0.197	2.4
Extracted with water at low temperature + dilute NaCl solution.................	26.6	0.665	12.72	0.411	4.16	0.095	0.095	0.570	1.38	0.190	0.205	2.0
After NaOH hydrolysis until Biuret-free................	33.9	0.847	16.88	0.545	4.58	0.104	0.104	0.743	1.36	0.248	0.272	2.4
After pancreatic digestion......	30.1	0.752	14.75	0.476	4.46	0.101	0.101	0.651	1.37	0.217	0.238	2.1
Ashed at low temperature (brown ash)................	34.2	0.855	16.85	0.543	4.40	0.100	0.100	0.755	1.39	0.251	0.271	2.5
Ashed at low temperature + NH₄NO₃ (white ash)........	35.5	0.887	17.07	0.551	3.76	0.085	0.085	0.802	1.45	0.267	0.275	3.1
Bone, inner portion (alcohol-ether-extracted)............	26.0	0.650	12.87	0.415	4.28	0.097	0.097	0.553	1.33	0.184	0.207	1.9
Calcium phosphate, dibasic (CaHPO₄·H₂O)............	24.9	0.622	20.37	0.657	0.27	0.006	0.006	0.616	0.94			
Calcium phosphate, tribasic, Preparation A (Ca₃(PO₄)₂)..	35.6	0.890	19.51	0.630	0.57	0.013	0.013	0.877	1.39	0.292	0.315	
Calcium phosphate, tribasic, Preparation B (Ca₃(PO₄)₂) (washed)................	33.09	0.827	17.07	0.551	3.53	0.080	0.080	0.747	1.35	0.249	0.275	3.1
Calcite, CaCO₃................	38.55	0.964	None	None	35.46	0.806	0.806					

It will be seen from the carbon dioxide analyses that there is an appreciable and relatively constant amount of carbonate present in bone. The mM of $CaCO_3$ are identical, of course, to mM of CO_2 found since they bear a 1:1 ratio to each other. The proportion of calcium held as carbonate agrees closely with the findings of Kramer and Shear (4) for old bone in rats and of Neal, Palmer, Eckles, and Gullickson (5) for mature cattle bone. Attention should be called to the trace of carbonate found in c.p. dibasic calcium phosphate, and more especially to the fact that commercial preparations of c.p. tertiary calcium phosphate contain appreciable but *varying* amounts of carbonate.

Residual calcium is calculated by subtracting the calcium held as $CaCO_3$ from the total Ca content, and should thus represent the calcium present in the form of phosphates. Hence, the ratio residual Ca:P is of special interest as indicating whether the chief inorganic salt in bone is secondary or tertiary calcium phosphate. On a molarity basis, Ca:P in the tertiary salt $(Ca_3(PO_4)_2)$ is 3:2 or 1.5; for secondary calcium phosphate $(CaHPO_4)$ this Ca:P ratio would be 1.0. It will be seen from Table II that the residual Ca:P ratio found for untreated bone is 1.47, closely approximating the theoretical 1.5 value. Variations from 1.36 to 1.76 were encountered in samples treated by various procedures. Taylor and Sheard (8) and Roseberry, Hastings, and Morse (6) found that the x-ray spectrograms of bone and tooth enamel agreed closely with those of members of the apatite series of minerals, but were unable to demonstrate the lines characteristic of secondary calcium phosphate.

In Columns 11 and 12 of Table II, are given the millimols of tertiary calcium phosphate which would be expected if all the residual calcium were present in this form (residual Ca:3), and if all the inorganic phosphates were tertiary calcium phosphate (P:2). Providing the above assumptions were completely true, and also that the analytical data were without error and the subtraction for calcium held as carbonate were exact, the mM of

residual Ca:P. This may be compared with the mean value 1.92 found by Howland, Marriott, and Kramer (2) and the value 1.99 found by Kramer and Shear (4). Neal, Palmer, Eckles, and Gullickson (5) found the weight ratio of $Ca_3(PO_4)_2:CaCO_3$ in untreated mature cattle bone to average 6.57, whereas our figure for this ratio on this basis is 6.37.

$Ca_3(PO_4)_2$ as calculated from residual Ca and as calculated from total inorganic P should be identical. It is surprising to see in what close agreement the values for $Ca_3(PO_4)_2$ calculated in these two ways are, especially since there is known to be a small amount of phosphorus held in combination with magnesium (probably as magnesium tertiary phosphate). This further confirms the belief that the bulk of the inorganic portion of bone is a tertiary calcium phosphate nucleus.

In Column 13, Table II, will be found the ratios for $Ca_3(PO_4)_2$: $CaCO_3$ for each of the ten preparations of bone, and also for the commercial preparation of pure $Ca_3(PO_4)_2$ designated as Preparation B. This ratio closely approximates 2.0 in the untreated bone and in those preparations where the composition is relatively unaltered by the treatment received. In this connection we may state that the main effect of the different processes to which the bone cortex was subjected seems to have been merely to increase the percentage of inorganic constituents slightly by removing some or all of the organic matter, leaving their relative proportions practically undisturbed. However, in ashed bone, and one specimen of $Ca_3(PO_4)_2$, Preparation B, the ratio of carbonate to phosphate molecules is more nearly 1:3. The other specimen of tertiary calcium phosphate analyzed (Preparation A) contained only small amounts of carbonate. These findings are in agreement with those of Roseberry, Hastings, and Morse (6) that the x-ray spectrograms of bone conform most closely to that shown by dahllite, the carbonate-phosphate which has the empirical formula $CaCO_3 \cdot 2Ca_3(PO_4)_2$. It is believed that in such a crystal the carbonate loses its individual identity, and that the chief inorganic constituent of bones seems to be neither tertiary calcium phosphate nor calcium carbonate, but a crystalline salt containing carbonate, phosphate, and calcium in definite space relations with one another.

The ratio of $Ca_3(PO_4)_2$: $CaCO_3$ approximates 2 in the specimens of untreated bone analyzed by us and in specimens not subjected to drastic experimental procedures. Other ratios varying between 2 and 3 have been observed in bones of different ages and under certain experimental conditions (2, 4).

SUMMARY

1. Analyses were made of the untreated outer and inner portions of bone, and of bone cortex treated in the following ways: ex-

tracted with alcohol, with ether, with water, hydrolyzed with NaOH, digested with pancreatin, and ashed at temperatures low enough to avoid loss of carbonates. These preparations were analyzed for their total calcium, inorganic phosphorus, and carbonate content.

2. The inner and outer portions of bone did not differ materially as to inorganic composition. The relative proportions of the inorganic constituents in bone cortex were undisturbed by the different processes to which it was subjected, except that ashing causes a considerable loss of carbonates unless special precautions are taken.

3. Commercial preparations of tertiary calcium phosphate as purchased contain appreciable but variable amounts of carbonate and hence are not uniform in composition. The residual Ca:P ratio of the two samples analyzed was the same but was slightly lower than the theoretical Ca:P ratio for that salt.

4. In all the bone preparations, the residual Ca:P ratio approximated that for tertiary calcium phosphate.

5. The $Ca_3PO_4:CaCO_3$ ratio varies between 2 and 3, but approximates 2 in all preparations where the bone substances have been relatively little altered by treatment.

6. The chief inorganic constituent of bone is probably a crystalline salt, $CaCO_3 \cdot nCa_3(PO_4)_2$, where n approximates the value 2 in untreated bone.

BIBLIOGRAPHY

1. Fiske, C. H., and Subbarow, Y., J. Biol. Chem., **66**, 375 (1925).
2. Howland, J., Marriott, W. McK., and Kramer, B., J. Biol. Chem., **68**, 721 (1926).
3. Kramer, B., and Tisdall, F. F., J. Biol. Chem., **47**, 475 (1921).
4. Kramer, B., and Shear, M. J., J. Biol. Chem., **79**, 147 (1928).
5. Neal, W. M., Palmer, L. S., Eckles, C. H., and Gullickson, T. W., J. Agric. Research, **42**, 115 (1931).
6. Roseberry, H. H., Hastings, A. B., and Morse, J. K., J. Biol. Chem., **90**, 395 (1931).
7. Shear, M. J., and Kramer, B., J. Biol. Chem., **79**, 105 (1928).
8. Taylor, N. W., and Sheard, C., J. Biol. Chem., **81**, 479 (1929).
9. Van Slyke, D. D., and Neill, J. M., J. Biol. Chem., **61**, 523 (1924).

THE PREPARATION OF VITAMIN C CONCENTRATES FROM LEMON JUICE*

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The present investigation of the preparation of concentrated antiscorbutic fractions from lemon juice has involved primarily a study of the solubility of the active material in organic solvents. Certain details in the general procedure previously published (1) have been modified in order to work satisfactorily with larger quantities of starting material. In view of the apparent difference in results obtained by workers of another laboratory (2) in attempting to make reprecipitations of the active factor with lead acetate and ammonia, this point has also been carefully rechecked and will be discussed in greater detail in a later paper.

The general procedure used in testing the vitamin C content of the various fractions was that recommended by Sherman, La Mer, and Campbell (3). In order to facilitate progress in fractionation, however, somewhat shorter test periods were used. The method employed for concentrating the antiscorbutic material from lemons[1] was approximately the same as that previously reported from this laboratory (1), and will be described in detail in the last section, including certain precautions found necessary when dealing with larger quantities of juice.

It will be noticed in the earlier tests in which control animals were fed a portion of the preparation after the second active precipitate was made, that it was regularly found that the equivalent of 1 cc. of lemon juice daily was sufficient to protect the animals from distinct scurvy. Mild scurvy occasionally resulted both on

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this preparation and on 1 cc. of the original juice. Therefore, no appreciable loss in activity occurred, because the daily feeding of a quantity of this second precipitate equivalent to 1 cc. of lemon juice (after deleading with ethyl alcohol and 1:3 HCl) was sufficient to prevent severe scurvy.

Fresh test preparations were made each week. After being freed from any toxic reagents or solvents used, they were made up to standard volume in slightly acidic aqueous solutions for feeding. All fractions to be assayed were stored under carbon dioxide in tightly stoppered flasks kept in a refrigerator, or in small vials (each of which contained enough of the preparation for daily feeding) placed in a desiccator with solid carbon dioxide.[2]

Solubility of Vitamin C Preparations in Organic Solvents

Starting with 400 cc. of fresh lemon juice, we prepared an absolute acetone solution of the active material according to the general method published previously. The steps involved were: decitration with basic lead carbonate and neutral lead acetate followed by precipitation with dilute ammonia, resolution with acetic acid, reprecipitation with ammonia, deleading with hydrochloric acid, extraction of waxes with butyl alcohol, addition of ethyl alcohol, evaporation almost to dryness, addition of absolute acetone, evaporation of the acetone solution to dryness, and extraction of the active material again with absolute acetone. The acetone solution (about 50 cc.) was then evaporated to 10 cc. and an equal volume of petroleum ether (freed from unsaturated hydrocarbons and distilled within a temperature range of 38–68°) was added. The results given in Table I show that the active factor is soluble in the 1:1 mixture of these two solvents. The total solids in the active fraction were decreased from 0.6 to 0.4 mg. per cc. of lemon juice by the last step. Some of the animals were gaining steadily on the soluble fraction at the end of the test.

It had been found previously in this laboratory that a significant amount of amorphous material was removed by extraction of the active solid material by ethyl ether, but the activity of the residue was uncertain. Vedder and Lawson (4) and Hart, Steenbock, and Lepkovsky (5) had reported that ethyl ether did not extract

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[2] We are indebted to the Dry Ice Corporation for supplying the entire amount required.

the active material from desiccated lemon juice, but they had not worked with analogous concentrates. The solubility of the vitamin has been found in our laboratory to vary greatly with changes in the nature of the material being extracted. An acetone solution of the active material from 400 cc. of lemon juice was

TABLE I

Solubility of Vitamin C in Petroleum Ether-Acetone (1:1)

Lemon juice equivalent fed daily (6 days per wk.)	No. of animals	Average survival (55 day test)	Average scurvy score*	Average gain in weight
		days		*gm.*
Soluble fraction, 2 cc................	4	55	6	−32
Insoluble " 2 "	5	25	18	−124
Both fractions, 2 cc. each.............	3	55	7	−48
Lemon juice, 1 cc......................	4	55	1	69
Basal diet only........................	4	24	18	−130

· The highest possible score is 24.

TABLE II

Extraction of Active Solid Material with Ethyl Ether

Lemon juice equivalent fed daily (6 days per wk.)	No. of animals	Average survival (55 day test)	Average scurvy score	Average gain in weight
		days		*gm.*
Soluble fraction, 3 cc...............	5	27	14	−136
Insoluble " 3 "	4	54	9	−119
Both fractions, 3 cc. each.............	5	46	6	−108
Second active ppt., deleaded with HCl and alcohol, 1 cc....................	3	55	4	52
Lemon juice, 1 cc......................	2	55	3	121
Basal diet only........................	3	21	14	−132

evaporated to dryness *in vacuo* and the residue extracted with carefully purified ethyl ether overnight. The results of feeding the various fractions are given in Table II. Vitamin C is apparently not soluble in ethyl ether, although this step reduced the total solids in the active fraction to 0.2 mg. per cc. of lemon juice. In view of the marked loss in activity in both fractions, it was

thought inadvisable to use ethyl ether in the further purification of the active material. Traces of peroxides which may be formed during the experiment would result in oxidation of the active factor.

Evidence has been previously given that vitamin C is probably acidic in nature (6). Dry ammonia gas passed into concentrated ethyl alcohol or ethyl acetate solutions of the active material cooled to 0° for the purpose of precipitating salts of acids present resulted in almost complete loss in activity, as is shown by the

TABLE III

Solubility of Vitamin C in Ethyl Acetate, and the Effect of Ammonia on Solutions of the Active Material in Organic Solvents

Lemon juice equivalent fed daily	No. of animals	Average survival (43 day test)	Average scurvy score	Average gain in weight
		days		*gm.*
Extraction of solid material with ethyl acetate				
Soluble　fraction, 2 to 4 cc.......	2	43	3	1
Insoluble　"　2 " 4 "　......	3	29	12	−146
Ethyl acetate solution of NH₃				
Soluble　fraction, 2 to 4 cc.......	5	29	14	−126
Insoluble　"　2 " 4 "	5	33	10	−115
Both fractions, 2 to 4 cc. each....	5	35	8	−110
Ethyl alcohol solution of NH₃				
Soluble　fraction 2 to 4 cc	5	32	13	−123
Insoluble　"　2 " 4 "　......	5	32	10	−130
Second active ppt., 1 cc..............	4	43	3	56
Lemon juice, 1 cc....................	2	43	3	69
Basal diet only.....................	2	26	14	−124

data in Table III. Saturated solutions of organic bases such as guanidine, urea, or choline (dissolved in a 1:1 petroleum ether-acetone mixture or in ethyl alcohol) produced no apparent effect when added to concentrated ethyl alcohol solutions of the active material. Extraction of the active solid material with absolute ethyl acetate for several hours showed clearly that the active material is soluble in this solvent. The same solvent had been previously reported not to extract vitamin C from desiccated lemon juice (5). The 2 cc. dosage was raised to 4 cc. on the 25th

day, following which the ethyl acetate-soluble fraction permitted a rapid resumption of growth, although this is not evident in Table III.

An active petroleum ether-acetone (1:1) solution of the active material prepared from 1500 cc. of lemon juice was evaporated to dryness *in vacuo* and the residue dissolved in *n*-butyl alcohol. The addition of petroleum ether in varying ratios produced a yellow flocculent precipitate on standing. The results of feeding the various fractions in this preparation, given in Table IV, indicate that vitamin C is soluble in 2:1 and 4:1 mixtures of petroleum

TABLE IV

Solubility of Active Factor in Butyl Alcohol and Petroleum Ether Mixtures

Lemon juice equivalent fed daily	No. of animals	Average survival (67 day test)	Average scurvy score	Average gain in weight
		days		*gm.*
Lemon juice, 1 cc.	2	67	0	179
Second active ppt., 1 cc.	5	67	1	136
Basal diet only	3	28	13	−111
Petroleum ether-butyl alcohol (2:1)				
Soluble fraction, 4 cc.*	3	67	2	10
Insoluble " 4 " *	5	29	14	−110
Petroleum ether-butyl alcohol (4:1)				
Soluble fraction, 4 cc.*	3	67	3	−15
Insoluble " 4 " *	5	30	16	−131
Both fractions, 4 cc. each*	2	67	2	97

* All 4 cc. levels were raised to 10 cc. after 30 days.

ether and *n*-butyl alcohol. The dosage of the various fractions was increased to 10 cc. at the end of 30 days in order to prevent the animals from losing weight. This required increase in dosage was thought to indicate at the time of the test that there had been a loss in the active material by oxidation but in view of later work, it is attributed to the fact that much of the active material had been removed at an earlier stage in the preparation. At the end of the test period, the animals receiving the soluble fractions were gaining steadily in weight.

In working with larger volumes of lemon juice, it became evident that the antiscorbutic factor was lost at some stage in the

preparation. Therefore, tests were made on the yellow syrupy residue which had been invariably thrown down by the addition of acetone to a concentrated solution of the active material obtained after deleading the second active precipitate. An absolute acetone solution of the active material from 2 liters of lemon juice was evaporated to dryness *in vacuo* and the residue extracted with 30 to 40 cc. of absolute ethyl acetate overnight. The data recorded in Table V clearly indicate that most of the activity was in the syrupy residue, although an appreciable amount was dissolved in the ethyl acetate. A trace of lead was found in the syrupy residue which was removed as lead phosphate by the addition of 20 per cent phosphoric acid before feeding this fraction.

TABLE V

Effect of Increasing Amount of Lemon Juice upon Extraction

Lemon juice equivalent fed daily	No. of animals	Average survival (43 day test)	Average scurvy score	Average gain in weight
		days		*gm.*
Lemon juice, 1 cc....................	1	43	0	83
Basal diet only......................	3	32	13	−188
Ethyl acetate solution, 10 cc..........	3	38	9	−130
Syrupy residue from addition of acetone, 10 cc.........................	4	43	0	208

It was evident from the results of the previous test that all traces of lead must be removed before the acetone was added, and that more care should be given to the extraction of the residues resulting from larger volumes of lemon juice. A second active precipitate was made from 2.5 liters of lemon juice. Lead was removed by treatment with 1:1 hydrochloric acid followed by ethyl alcohol. The alcohol solution was concentrated to 5 cc. *in vacuo* (12 to 14 mm.) at a bath temperature of 70–80°. Small amounts of hydrochloric acid were added if the liquid did not remain strongly acidic, and 15 volumes of acetone were added. A white semicrystalline mass was thrown down which was extracted twice with small amounts of acetone. The acetone solution was evaporated to dryness *in vacuo* at a bath temperature of 70–80° in the presence of very fine, carefully purified quartz and the dark-

ened residue extracted with absolute acetone overnight. The residue was again extracted with fresh portions of acetone for at least 5 hours. The solubility of the active material in ethyl acetate and petroleum ether-propyl alcohol mixtures was also studied. The results of the tests, given in Table VI, indicate clearly that vitamin C is soluble in these solvents, and that the modified procedure for the acetone extraction resulted in much less loss at this point.

TABLE VI

Solubility of Active Material in Ethyl Acetate and Petroleum Ether-Propyl Alcohol Mixtures

Lemon juice equivalent fed daily	No. of animals	Average survival (60 day test)	Average scurvy score	Average gain in weight
		days		*gm.*
Lemon juice, 1 cc.....................	2	60	4	63
Basal diet only........................	3	33	18	−114
Extraction of active solid material with ethyl acetate				
Soluble fraction, 10 to 5 cc........	3	60	4	110
Both fractions, 10 to 5 cc. each....	4	60	1	207
Petroleum ether-propyl alcohol (1:1)				
Soluble fraction, 10 to 5 cc......	3	60	0	238
Insoluble " 10 " 5 "	4	60	17	−32
Both fractions, 10 to 5 cc. each...	4	60	0	242
Petroleum ether-propyl alcohol (3:1)				
Soluble fraction, 10 to 5 cc......	4	60	5	145
Insoluble " 10 " 5 "	4	60	18	−40
Both fractions, 10 to 5 cc. each...	3	60	2	210
Solids not soluble in acetone, 10 to 5 cc..	3	60	19	24

In order to differentiate better between the activities of the various fractions, the dosage was decreased from 10 to 5 cc. at the end of 35 days. The advantage gained was evident from the trend of the weight curves after 35 days and from the final scores. Most of the activity was retained in solution although significant amounts were present in the residues. All animals receiving the insoluble residues were losing weight rapidly at the end of the 5 cc. period, although those receiving the soluble fractions were still gaining. The total activity of the various fractions being con-

sidered, there appears to have been no great loss in activity of the antiscorbutic fractions, even though the temperatures for distillation were higher than those previously used, the time for distillations was increased, and each of the completed preparations was fed for a period of 7 days. The final preparations were stored in small vials placed with dry ice in an insulated desiccator.

The final active preparations obtained showed strong reducing properties and were distinctly acidic. Carbylamine and phenolic tests were negative. Strongly positive tests were given with orcinol (blue-green) and resorcinol (pink).

SUMMARY

Vitamin C has been shown to be soluble in petroleum ether-acetone (1:1), petroleum ether-butyl alcohol (2:1 and 4:1), petroleum ether-propyl alcohol (1:1 and 3:1), ethyl acetate, butyl alcohol, and propyl alcohol, but insoluble in absolute ethyl ether.

Ammonia gas destroys the active material when passed into solutions containing the vitamin dissolved in organic solvents.

Details have been worked out for the preparation of active fractions from volumes of lemon juice up to 2.5 liters. The most concentrated preparations yet obtained (0.03 to 0.5 mg. of total solids per cc. of lemon juice) are sufficiently stable to be kept for weekly feeding periods and have given no indication of more than one active factor being involved. The active material was consistently characterized by being distinctly acidic and exerted a strong reducing action.

BIBLIOGRAPHY

1. Grettie, D. P., and King, C. G., *J. Biol. Chem.*, **84**, 771 (1929). Sipple, H. L., and King, C. G., *J. Am. Chem. Soc.*, **52**, 420 (1930).
2. Zilva, S. S., *Biochem. J.*, **24**, 1687 (1930); **25**, 594 (1931).
3. Sherman, H. C., La Mer, V. K., and Campbell, H. L., *J. Am. Chem. Soc.*, **44**, 165 (1922).
4. Vedder, E. B., and Lawson, W. E., *J. Biol. Chem.*, **73**, 215 (1927).
5. Hart, E. B., Steenbock, H., and Lepkovsky, S., *J. Biol. Chem.*, **52**, 241 (1922).
6. McKinnis, R. B., and King, C. G., *J. Biol. Chem.*, **87**, 615 (1930).

THE PREPARATION AND STORAGE OF VITAMIN C CONCENTRATES FROM LEMON JUICE*

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The object of this investigation has been to study (a) the conditions necessary for obtaining precipitation of vitamin C with basic lead acetate, (b) the use of n-butyl alcohol as a solvent, and (c) the stability of purified preparations stored in the laboratory.

The general method employed for concentrating and assaying the antiscorbutic factor from lemons[1] was the same as that previously reported from this laboratory (1–3). There has been no difficulty in obtaining almost complete recovery of activity by a second precipitation with lead by any of five independent workers in our laboratory over a period of several years, but it is evident from the experience of another laboratory (4) that using somewhat different reagents does not lead to such recovery. The pH of the solutions from which active precipitates were obtained has been carefully rechecked in confirming our earlier reports. Practically the only change in procedure found necessary to avoid serious loss in the activity of preparations has been greater care in regard to the mechanical condition of residues from which the vitamin is extracted by organic solvents (2).

·Fresh test preparations were made weekly, which, after being freed from toxic reagents or solvents used, were made up to a standard volume in slightly acidic aqueous solution, placed in vials containing sufficient quantities for 1 day's feeding, stoppered securely, and stored either in a refrigerator under carbon dioxide or nitrogen gas or in a desiccator with solid carbon dioxide.[2]

* Contribution No. 219 from the Department of Chemistry, University of Pittsburgh.

[1] We are indebted to the California Fruit Growers Exchange for providing a comparatively uniform supply of lemons.

[2] We are indebted to the Dry Ice Corporation for supplying the entire amount required.

Storage experiments clearly indicated that the purified preparations can be held for a period of 2 or 3 weeks without serious loss of antiscorbutic value when properly protected from oxidation during laboratory manipulation and subsequent storage.

EXPERIMENTAL

1½ liters of fresh lemon juice were decitrated, and an absolute acetone solution of the active material was prepared according to the general method previously published (2). The acetone solution was then evaporated to dryness and the residue extracted with 2 cc. of n-butyl alcohol, evaporated to 0.5 cc., cooled to 0°, and

TABLE I

Vitamin C Concentrate Obtained with n-Butyl Alcohol

Test preparations	No. of animals	Fed daily, lemon juice equiv- alent	Average survival (60 day test)	Average scurvy score	Average gain in weight
		cc.	*days*		*gm.*
Butyl alcohol solution............	5	10	60	4	7
" " "	4	20	60	2	145
White ppt........................	4	10	37	14	−137
" "	3	20	36	14	−220
" " + butyl alcohol solution	2	10	60	3	23
Second active ppt................	5	1	60	0	203
Positive controls................	4	1	60	0	244
Negative "	4	0	33	19	−144

centrifuged. The liquid and solid phases were then made up to standard volume and assayed (3). All evaporations were carried out *in vacuo* and in an atmosphere of carbon dioxide gas. The results given in Table I show that the active factor is soluble in *n*-butyl alcohol in the absence of water. The high feeding level (10 to 20 cc.) required was found later to be largely due to loss of activity during the acetone separation coincident with the increased volume of juice used (2). The amount of total solids in the active fraction was 0.09 mg. per cc. equivalent of lemon juice and consisted of acidic organic material which showed a reducing value equivalent to that of glucose (except more active qualitatively).

In view of the fact that the active preparations were always characterized by a high content of strongly reducing substances and acidic material analogous to the "hexuronic acid" isolated by Szent-Györgyi (5), it was thought desirable to find whether the use of a strongly reducing, weak acid, such as formic, would prove advantageous in protecting the concentrates. Since citric acid frequently accompanies vitamin C, and does not cause rapid decomposition of uronic acids (in contrast to hydrochloric acid), it was compared with the other two. Concentrated preparations were made separately in which the three acids were used com-

TABLE II

Storage Test (14 Days) on Absolute Acetone Concentrate from Lemon Juice

Test preparations	No. of animals	Fed daily, lemon juice equivalent	Average survival (72 day test)	Average scurvy score	Average gain in weight
		cc.	*days*		*gm.*
Absolute acetone extract					
Fed without storage............	3	10–5*	72	0	197
Stored 14 days in ice box........	4	10–5*	72	2	200
" 14 " " dry ice........	4	10–5*	72	0	218
Absolute acetone residue..........	3	10–5*	72	0	303
Aqueous " "	3	10	68	18	−170
Positive controls.................	4	1	72	0	198
Negative "	3	0	34	17	−200

* The lemon juice equivalent was reduced from 10 cc. to 5 cc. on the 46th day.

paratively during evaporations, storage, etc., and each preparation was then made up to standard volume with water acidified with the acids and divided into three parts. The first part was fed without storing, the second was stored in an ice box for 1 week, and the third was stored in dry ice for 1 week before starting the feeding tests. The results showed that citric and formic acids were no better for use in the general procedure or during storage than hydrochloric acid. No significant loss in activity was found to result from the storage.

Evidence from the preceding experiment pointed to the possi-

bility of a longer storage period for vitamin C concentrates than we had previously used. Such a condition would permit more time in the preparation of active fractions and greater freedom in feeding tests.

2½ liters of fresh lemon juice were decitrated and the absolute acetone solution obtained by the above general procedure. The solution was evaporated to dryness *in vacuo*, made up to standard

TABLE III

pH Values of Solutions from Which Active Precipitates Were Obtained

Cc. of NH₄OH	pH	Change in solution	Brom-thymol blue	Cresol red	Phenol red	Thymol blue
First active precipitate						
3	5.37	None	Yellow	Yellow	Yellow	Yellow
6	5.54	"	"	"	"	"
8	6.3	Yellow	Yellow-green	"	"	"
9	7.5*	" ppt.	Blue	Orange	Red	"
12	8.8	Slight "	"	Orange-red	Dark red	Blue-green
Second active precipitate						
	3.4		Yellow	Yellow	Yellow	Yellow
50.5	6.1	Yellow	Yellow-green	"	"	"
52	6.4	Ppt.	Green	"	Orange	"
55.5	7.3*	"	Blue-green	Yellow-orange	Pink	"
56	8.0	Slight ppt.	Blue	Orange	Red	Yellow-green

* Addition of ammonia was regularly stopped at this point in the preparation of active concentrates.

volume with water acidified with hydrochloric acid, and divided into three parts. The first part was placed in vials, each containing enough of the preparation for 1 day's feeding, placed under an atmosphere of nitrogen, and fed during the ensuing week without preliminary storage; the second part was similarly placed in vials and stored in an ice box for 14 days under an atmosphere of nitrogen before commencing the feeding period; and the third part was

stored 14 days under an atmosphere of carbon dioxide gas with dry ice in a desiccator before starting the feeding period. The results, recorded in Table II, show that the antiscorbutic value of the concentrates was well conserved during storage in both dry ice and the ice box. A similar test carried out simultaneously on an ethyl acetate-soluble concentrate showed very little loss during 14 days storage, with some advantage in favor of the dry ice. The latter product seemed to furnish an ideal storage medium because of the low temperature, inert atmosphere, and slight acidity. Since practically complete protection against scurvy was shown by both the acetone-soluble fraction and the acetone residue on a 5 cc. feeding level, it is evident that complete extraction with acetone would have afforded protection on a 2.5 cc. level.

Careful tests were made to determine the pH value of our solutions during the first and second precipitations with lead acetate and ammonia, in view of the contrasting results obtained when a somewhat different procedure in another laboratory was followed (4). The results given in Table III show that the point where complete precipitation of the active principle occurred was at or near pH 7.5. Relatively good agreement was regularly found between the quinhydrone or hydrogen electrode indication of pH values and those obtained by the use of phenol red.

SUMMARY

An antiscorbutic concentrate was obtained by extracting the solids from an absolute acetone solution with n-butyl alcohol and cooling until an inactive precipitate separated. The active extract contained 0.09 mg. per cc. of lemon juice equivalent. It reduced cold potassium permanganate and ammoniacal silver nitrate readily, and gave a strongly positive test with orcinol and resorcinol. Tests for phenols were negative. The reducing value was equivalent to glucose.

It was shown that repeated active precipitates were thrown down at a pH of 7.3 to 7.5, as indicated both by quinhydrone and hydrogen electrodes and standard indicators.

Citric and formic acids were found to be no better than hydrochloric acid for the protection of the active factor during the preparation or storage of vitamin C concentrates.

The purified preparations can be held for a period of 2 to 3 weeks

without serious loss of activity if properly protected from oxidation during laboratory manipulations and subsequent storage by the use of an atmosphere of nitrogen or carbon dioxide. Dry ice proved to be particularly suitable. The improved stability is apparently due in part to separation from the enzymes originally present, although the use of anaerobic acid solutions and specially prepared reagents has probably been a contributing factor.

BIBLIOGRAPHY

1. Grettie, D. P., and King, C. G., *J. Biol. Chem.*, **84**, 771 (1929). Sipple, H. L., and King, C. G., *J. Am. Chem. Soc.*, **52**, 420 (1930).
2. Svirbely, J. L., and King, C. G., *J. Biol. Chem.*, **94**, 483 (1931).
3. Sherman, H. C., La Mer, V. K., and Campbell, H. L., *J. Am. Chem. Soc.*, **44**, 165 (1922).
4. Zilva, S. S., *Biochem. J.*, **24**, 1687 (1930); **25**, 594 (1931).
5. Szent-Györgyi, A., *J. Biol. Chem.*, **90**, 385 (1931); *Biochem. J.*, **22**, 1387 (1928).

RELATIONSHIPS BETWEEN THE STRUCTURE OF SATURATED ALIPHATIC ALCOHOLS AND THEIR INHIBITING EFFECT UPON LIVER ESTERASE*

By DAVID GLICK and C. G. KING

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The study of enzyme kinetics has led to the conception of active centers or special chemical groups in complex organic substances of protein-like nature which constitute the common enzymes. To what degree the activity of such catalysts depends upon the surface energy forces of the colloidal particles is largely undetermined, but it seems likely from the study of enzyme kinetics and from consideration of the chemical and stereochemical specificity of enzymes, that unstable intermediate compounds are formed by union of an active group with the substrate. The products of the reaction catalyzed or other added substances may also form combinations with the active catalytic group and thus set up a series of reversible equilibria as shown by Michaelis and Menten (1) and others (2).

Such varied types of compounds as alkaloids (3), chloroform, iodoform, and formaldehyde (4), ketones (5), ketocarboxylic acid esters (6), and secondary alcohols (7) have been shown to combine with esterases and lipases to inhibit their activity, presumably by the formation of enzyme-inhibitor complexes which effectively reduce the enzyme concentration available for combination with the substrate. The specificity of esterases for optical isomers (7–10) indicates strongly that there are certain optically active groups in the enzyme by which such combinations are made. Except the indirect evidence of an enolic structure given by Falk (11), practically nothing has been reported or conjectured regarding the chemical nature of the organic groups which constitute the active

* Contribution No. 221 from the Department of Chemistry, University of Pittsburgh.

centers in esterases. From the general characteristics of esterases, and from analogy to other organic reactions, it seems probable that there is involved in the formation of enzyme-substrate and enzyme-inhibitor compounds at least two factors; first, that of molecular attraction as exhibited by the dissolving of one substance in another, and second, the formation of definite combinations of an unstable nature, analogous to the oxonium compounds or double salts. The latter type of reaction would be similar to salt formation through secondary or unsatisfied valences and would involve the reactive groups, such as ketones, aldehydes, alcohols, nitriles, and organic halides.

On this basis, the affinity of the enzyme for other substances should furnish some indication of the nature of the enzyme itself; *i.e.*, it would be reasonable to assume the presence of groups in the enzyme which would both adsorb (by physical attraction forces) and combine with (chemical reaction) suitable substrates or inhibitors. The marked increase in the affinity of an esterase for the members of a series of substrates or inhibitors with greater length of hydrocarbon chain, such as reported in this paper, may be taken to illustrate the first factor mentioned above. This may also be, an indication of the presence of a long chain hydrocarbon group in the active portion of the enzyme structure. Illustrative of the second factor, the accessibility of the reactive group in the inhibitor should govern the degree of inhibitory power shown by a series of compounds, such as the aliphatic alcohols herein studied, which have the reactive group masked in varying degrees by adjacent groups. The experimental work included in the present paper is interpreted as being at least in harmony with the above point of view. Considerations of the chemical nature of the active parts of enzymes are admittedly highly speculative from any evidence now available.

A third obvious factor in considering the effect of inhibitors upon enzyme activity is the effect which may be produced upon the degree of dispersion of the protein complex characteristic of the common enzymes. This phase of the problem has been studied extensively in relation to the effect of electrolytes (12) but not in relation to the effect of small amounts of organic substances.

Preparation of Materials—Fresh lamb liver was treated with acetone to remove fat and water, according to the method of Willstätter and Waldschmidt-Leitz (13). The powdered dry preparation was stored in a refrigerator with no marked loss in activity over a period of several months. Extractions were made from 0.5 gm. of this preparation with 15 cc. of 0.025 N NH₄OH for 3 hours at 37° with occasional shaking. After the residue had been centrifuged off, the solution was neutralized with 0.1 N CH₃COOH, methyl red being used as an outside indicator. After being centrifuged, the clear liquid was made neutral to brom-thymol blue, used as an outside indicator, with ammonia. Active extracts kept in a refrigerator and removed only when needed could be used for at least a week without loss in activity. Preparations made with the above reagents were more active than those obtained when NaOH or HCl was substituted for NH₄OH or CH₃COOH.

The brom-thymol blue indicator was prepared by dissolving 0.1 gm. of the solid in 1 liter of water. At the concentration used, this indicator exerts very little effect upon the enzyme (14). An aqueous rather than the usual alcoholic solution of brom-thymol blue was used since it has been found that, under the conditions of the experiment, alcohol in the indicator may produce about 15 per cent inhibition (15). The stock solution of substrate contained 4.6440 gm. of pure ethyl butyrate per liter (0.040 M). Carbon dioxide-free water, freshly distilled from glass, was used for all reagents and for rinsing glassware.

The purified alcohols were obtained from the Eastman Kodak Company. Before use, the boiling points were rechecked, and when necessary, the alcohols were distilled and the proper fractions collected and used. When the aqueous solutions were turbid, they were diluted to known volumes until perfectly clear. Alcohols containing an asymmetric configuration were used only in the form of pure racemic mixtures.

Procedure—The method of measuring the velocity of hydrolysis depended upon the direct titration of butyric acid formed in a buffer-free medium (9, 16, 17). A burette delivering 80 drops per cc. was used and each run, with six tubes in a series, was com-

pleted when 40 drops of 0.01 N NaOH had been added to the control tube. All experiments were conducted at room temperature. An extreme change of 10° was found to have no significant effect upon the relative velocities of hydrolysis, although the absolute velocities were changed. Ethyl butyrate solution (1.0 cc.) was added to each test-tube filled to a final volume of 8 cc. (0.005 M), since this concentration of substrate gave the maximum velocity of hydrolysis.

DISCUSSION

Inhibition Number—To simplify comparison of the inhibitory powers of various substances, the term "inhibition number" is

TABLE I

Inhibition of Normal Primary Alcohols on Sheep Liver Esterase

Curve No.	Alcohol	Mols $\times 10^{-6}$ for 25 per cent inhibition	Inhibition No.
I	Methyl	436.0	1.0
II	Ethyl	143.0	3.1
III	Propyl	53.0	8.2
IV	Butyl	37.5	11.6
V	Amyl	9.0	48.4
VI	Hexyl	4.9	89.0
VII	Heptyl	1.8	242.0
VIII	Octyl	0.9	459.0
IX	Nonyl	0.5	838.0

introduced, which is defined as the ratio of the number of mols of methyl alcohol needed to produce 25 per cent inhibition (with certain concentrations of substrate, enzyme, etc.) to the number of mols of another substance needed to produce the same inhibition under the same conditions. Thus from data in Table I, it takes 436×10^{-6} mols of methyl alcohol to produce 25 per cent inhibition, while it requires 143×10^{-6} mols of ethyl alcohol to give the same inhibition. Hence the ratio $\frac{436}{143}$ or 3.05 is the inhibition number of ethyl alcohol. The value of the term lies in the fact that its magnitude is a direct measure of the inhibiting power of the substance in question referred to the standard, methyl alcohol. These

values enable one to compare immediately the inhibitory powers of substances without reference to the original curves.

Normal Primary Alcohols—The inhibitions of the normal primary alcohols from methyl to nonyl inclusive are given in Figs. 1 and 2. It is evident that as the hydrocarbon chain is lengthened, the inhibitory power rises rapidly. Saturated solutions of
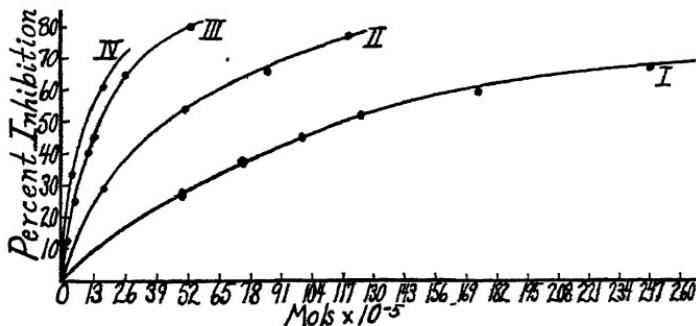
FIG. 1. Inhibition by aliphatic alcohols. Curve I, methyl alcohol; Curve II, ethyl alcohol; Curve III, propyl alcohol; Curve IV, butyl alcohol.
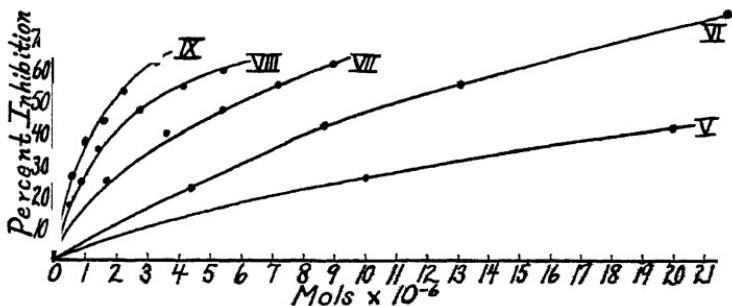
FIG. 2. Inhibition by aliphatic alcohols. Curve V, amyl alcohol; Curve VI, hexyl alcohol; Curve VII, heptyl alcohol; Curve VIII, octyl alcohol; Curve IX, nonyl alcohol.

lauryl and myristyl alcohols at 25° showed some inhibition, but that of cetyl alcohol gave none, due to its extreme insolubility in water.

Isomers of Amyl Alcohol—From the data in Table II and Fig. 3, it is evident that as the steric hindrance about the OH group is increased, the tendency of the inhibitor to combine with the

TABLE II

Inhibition of Isomeric Amyl Alcohols on Sheep Liver Esterase

Curve No.	Alcohol	Formula	Mols × 10⁻⁶ for 25 per cent inhibition	Inhibition No.
A	n-Amyl alcohol	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot OH$	9.0	48.4
B	Isoamyl alcohol	$CH_3 \cdot CH \cdot CH_2 \cdot CH_2 \cdot OH$ $\quad\quad \overset{\cdot}{C}H_3$	17.0	25.7
C	Secondary butyl-carbinol	$CH_3 \cdot CH_2 \cdot CH \cdot CH_2 \cdot OH$ $\quad\quad\quad \overset{\cdot}{C}H_3$	20.0	21 8
D	n-Propylmethyl-carbinol	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH \cdot OH$ $\quad\quad\quad\quad \overset{\cdot}{C}H_3$	40 0	10.9
E	Diethylcarbinol	$CH_3 \cdot CH_2 \cdot CH \cdot OH$ $\quad\quad\quad \overset{\cdot}{C}H_2$ $\quad\quad\quad \overset{\cdot}{C}H_3$	55.0	7.9
F	Isopropylmethyl-carbinol	$\quad\quad CH_3$ $CH_3 \cdot \overset{\cdot}{C}H \cdot CH \cdot OH$ $\quad\quad\quad\quad \overset{\cdot}{C}H_3$	83.0	5.2
G	Tertiary amyl alcohol	$\quad\quad CH_3$ $CH_3 \cdot CH_2 \cdot \overset{\cdot}{C} \cdot OH$ $\quad\quad\quad \overset{\cdot}{C}H_3$	315.0	1.4
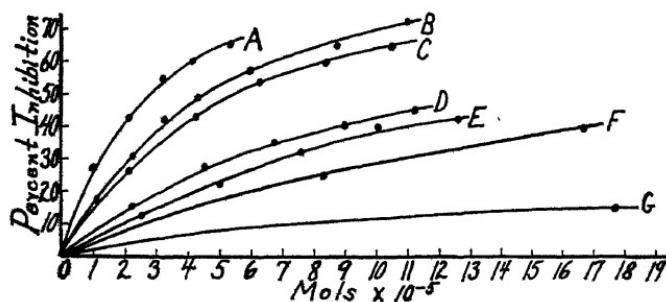
FIG. 3. Inhibition by isomers of amyl alcohol. Curve A, amyl alcohol; Curve B, isoamyl alcohol; Curve C, secondary butylcarbinol; Curve D, n-propylmethylcarbinol; Curve E, diethylcarbinol; Curve F, isopropyl-methylcarbinol; Curve G, tertiary amyl alcohol.

TABLE III

Inhibition of Secondary Alcohols on Sheep Liver Esterase

Curve No.	Alcohol	Formula	Mols × 10^{-6} for 25 per cent inhibition	Inhibition No.
A	Methyl-β-phenylethyl-carbinol	$CH_2 \cdot CH_2 \cdot CH \cdot OH$ CH_3	2.8	156.0
I	Phenylethylcarbinol	$CH_3 \cdot CH_2 \cdot CH \cdot OH$	4.5	96.9
B	Phenylmethylcarbinol	$CH_3 \cdot CH \cdot OH$	10.5	41.5
II	Diethylcarbinol	$CH_3 \cdot CH_2 \cdot CH \cdot OH$ CH_2 CH_3	55.0	7.9
III	Secondary butyl alcohol	$CH_3 \cdot CH_2 \cdot CH \cdot OH$ CH_3	230.0	1.9
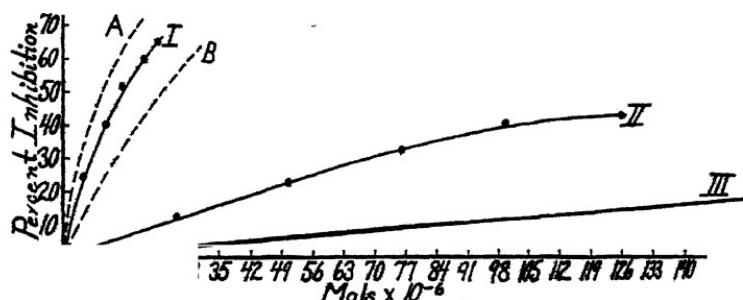
Fig. 4. Inhibition by secondary alcohols. Curve A, methyl-β-phenyl-ethylcarbinol; Curve I, phenylethylcarbinol; Curve B, phenylmethyl-carbinol; Curve II, diethylcarbinol; Curve III, secondary butyl alcohol.

enzyme becomes less, as is evidenced by the decrease in the inhibition number. This seems to indicate that the OH group is a point of attachment of the inhibitor to the enzyme. This also may be taken to indicate the chemical nature of the reaction forming an inhibitor-enzyme complex, since the same limitation of steric hindrance exists here which affects the normal chemical reactions of the isomeric alcohols. Aside from the chemical affinity, however, the possibility must be kept in mind that structures producing the greatest inhibitions may be those exerting the greatest effect upon the interfacial tensions with the enzyme surface.

Other Secondary Alcohols—Reference to Table III and Fig. 4 shows that although steric hindrance operates to decrease the inhibitory power, it is overshadowed by the effect of enlarging the hydrocarbon part of the molecule. Thus phenylethylcarbinol has an inhibition number of 96.9 while that of secondary butyl alcohol is only 1.9 in spite of the fact that the steric hindrance effect in the former compound is greater than that in the latter. The data for Curves A and B are taken from Murray and King (7).

<div align="center">SUMMARY</div>

The term inhibition number has been suggested for expressing the relative inhibitory powers of various substances.

The inhibitions (of sheep liver esterase acting on ethyl butyrate) produced by normal primary alcohols have been measured and found to increase rapidly as the length of the hydrocarbon chain is increased.

Likewise the inhibitions of seven isomers of amyl alcohol have been measured and found to decrease as the steric hindrance about the OH group increases.

The inhibitory effects of several secondary alcohols were determined and the effect of the size of the hydrocarbon group was found to be greater than that of nominal steric hindrance.

Some of the factors relating to the effects of organic inhibitors are discussed briefly.

<div align="center">BIBLIOGRAPHY</div>

1. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
2. Haldane, J. B. S., Enzymes, New York and London, 38–49, 80–92 (1930).

3. Rona, P., and Pavlovic, R., *Biochem. Z.*, **130**, 225 (1922).
4. Palmer, L. S., *J. Am. Chem. Soc.*, **44**, 1527 (1922).
5. Murray, D. R. P., *Biochem. J.*, **23**, 292 (1929).
6. Willstätter, R., Kuhn, R., Lind, O., and Memmen, F., *Z. physik. Chem.*, **167**, 303 (1927).
7. Murray, D. R. P., and King, C. G., *Biochem. J.*, **24**, 190 (1930).
8. Ammon, R., *Fermentforschung*, **11**, 459 (1930).
9. Bamann, E., *Ber. chem. Ges.*, **62**, 1538 (1929).
10. Rona, P., and Ammon, R., *Biochem. Z.*, **181**, 49 (1927).
11. Falk, K. G., *Proc. Nat. Acad. Sc.*, **2**, 557 (1916).
12. Sherman, H. C., Caldwell, M. L., and Cleaveland, M., *J. Am. Chem. Soc.*, **52**, 2436 (1930).
13. Willstätter, R., and Waldschmidt-Leitz, E., *Z. physiol. Chem.*, **125**, 132 (1923).
14. Bamann, E., and Schmeller, M., *Z. physiol. Chem.*, **194**, 1 (1931).
15. Murray, D. R. P., *Biochem. J.*, **24**, 1890 (1930).
16. Bamann, E., and Schmeller, M., *Z. physiol. Chem.*, **183**, 149 (1929).
17. Knaffl-Lenz, E., *Arch. exp. Path. u. Pharmakol.*, **97**, 242 (1923).

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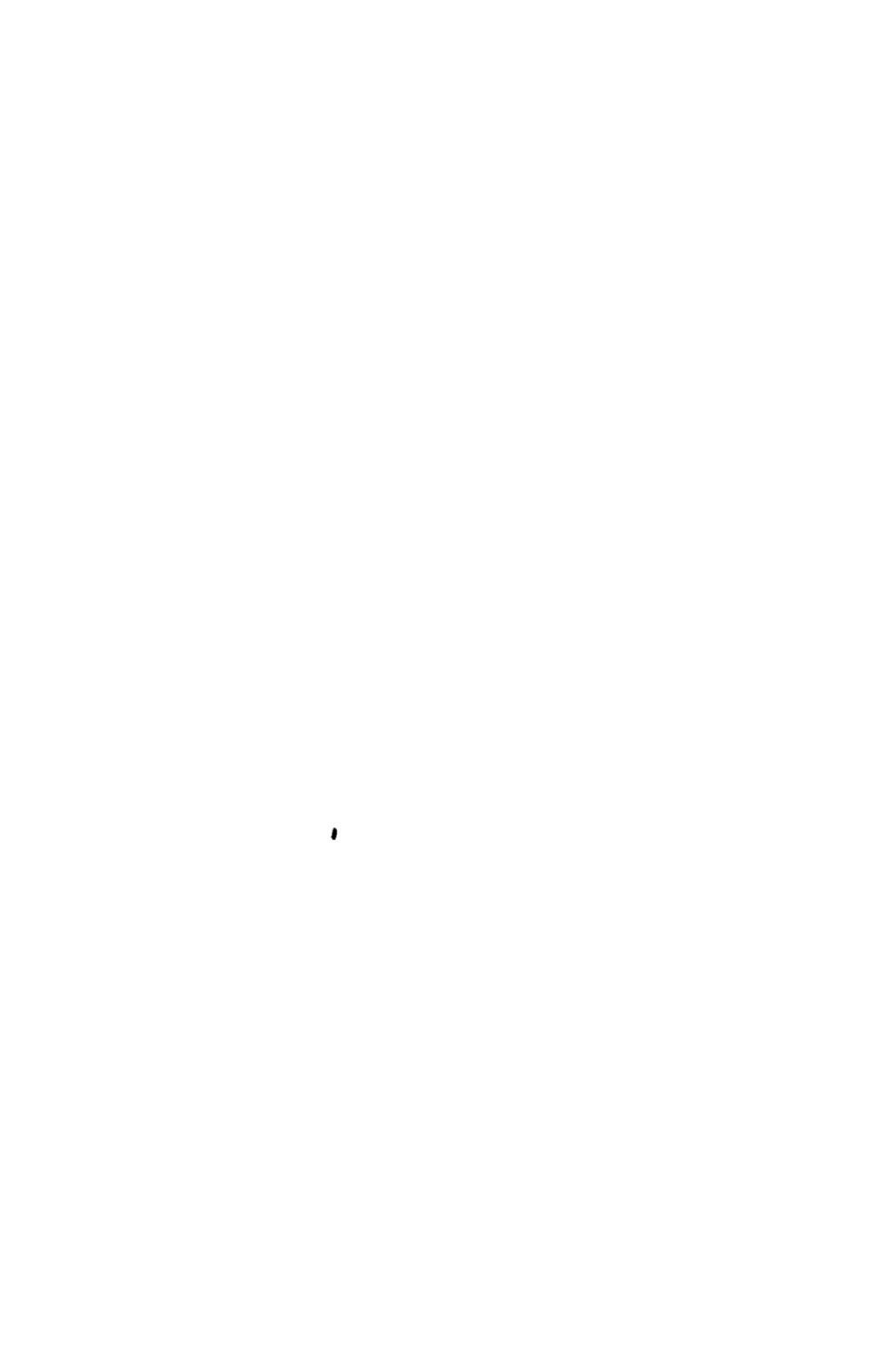
A PIPETTE FOR THE HANDLING OF WHOLE BLOOD SAMPLES, FOR USE WITH THE VAN SLYKE GASOMETRIC APPARATUS

By GEORGE MARTIN GUEST

(From the Children's Hospital Pediatric Research Foundation, and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)

In making gasometric determinations on whole blood, when it is necessary to take blood samples at a distance from the laboratory, difficulty is often experienced in obtaining even mixtures of the cells and plasma for analysis. If the blood when drawn is delivered into a centrifuge tube under oil, mixed with an anticoagulant (or defibrinated), and carried to the laboratory, the cells settle out slightly and it is difficult to stir them so as to obtain again an even suspension; the stirring is uncertain and is especially troublesome with small samples of blood because droplets of oil are stirred into the blood. The pipette described here was devised to overcome this difficulty.

The pipette[1] shown in Fig. 1 is made from a 2-way stop-cock with 2.0 mm. capillary tubes. The holes of the cock should fit exactly the openings of the upper and lower delivery tubes. Bulbs are blown in the tubes as shown, and the lower bulb A is calibrated by weighing mercury *to contain* 1.0, 0.5, or 0.2 ml. from the bottom of the stop-cock to the tip of the tube; the upper bulb B is calibrated by weighing water *to deliver* 1.0 ml. from the bottom of the stop-cock to a mark above the bulb.

To prepare the pipette for use in the CO_2 analysis, CO_2-free water is drawn through A and B to the mark above B, care being taken that no air bubbles are caught at the capillary of the cock. The cock is then turned to deliver through the side arm C, and the

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[1] This pipette has been made for us by the Central Scientific Company, Chicago.

bulb A and side arm C are dried by suction, alcohol and ether being used as usual.

A blood sample is delivered into a centrifuge tube under oil, and mixed with an anticoagulant or carefully defibrinated by turning a glass rod in the blood. The tip of the pipette is then thrust through the layer of oil into the blood and the blood is drawn by gentle suction to slightly above the stop-cock in the side arm C.
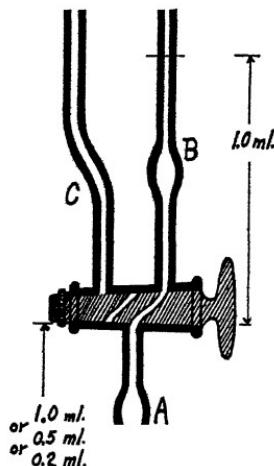
FIG. 1.   Bulb A is calibrated *to contain*; bulb B is calibrated *to deliver*

The cock is then turned at a right angle, automatically cutting off the exact sample in the bulb A, and a rubber band is then placed over the tip of the bulb A and around the hub of the stop-cock. The pipette may be carried thus to the laboratory (preferably in an upright position), or without a rubber band the pipette may be thrust into a test-tube filled with mercury, which effectively closes the lower end. The mercury may be chilled to stop enzyme changes in the blood.

For the delivery of the sample into the Van Slyke manometric

apparatus, the cup of the apparatus receives the usual amount of CO_2-free water, less 1.0 ml., and the tip of the pipette is inserted into the cup under this layer of water. The cock is then turned to deliver from the bulb B, and as the blood is delivered the water from bulb B rinses the blood from the lower bulb. Usually a few blood cells stick to the sides of the tube, but certainly in no greater number than remain when there is no rinsing; when dealing with concentrated blood (in anhydremia) the delivery is much more complete than it is from the ordinary pipette.

For oxygen analysis, the same procedure is applicable with necessary modifications of the reagents.

ANIMAL CALORIMETRY

FORTY-FIRST PAPER

THE INFLUENCE OF PHLORHIZIN GLYCOSURIA ON THE METABOLISM OF DOGS AFTER THYROIDECTOMY*

BY MARGARET DANN, WILLIAM H. CHAMBERS, AND GRAHAM LUSK

WITH THE TECHNICAL ASSISTANCE OF JAMES EVENDEN

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In 1912, when Carl von Noorden visited this country, he pointed out to workers in this laboratory that the increases in protein metabolism and in total heat production, which are the rule after the administration of phlorhizin to dogs, would not take place if the thyroid gland were first removed. His own opinion, elsewhere stated, was that "the thyroid acts upon metabolism as a pair of bellows acts upon a fire." Lusk (1) at the Seventeenth International Congress of Medicine, held at London, in 1913, presented evidence of the truth of von Noorden's conception. The experiments then cited have never been published in full, and these, together with other experiments of more recent date, form the material presented in this paper.

HISTORICAL

Minkowski (2), in 1896, removed the thyroid and pancreas from dogs either in a single operation or in two successive operations, in which pancreatectomy was followed by thyroidectomy, and he declared that the absence of the thyroid had no influence either on the course of pancreas diabetes or on the process of sugar formation. He also stated that phlorhizin diabetes followed its usual course in thyroidectomized dogs. Eppinger, Falta, and Rudinger (3), in 1908, performed thyroidectomy on a dog

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weighing 35 kilos and pancreatectomy 2 weeks later. The urine of this dog maintained an average D:N ratio of 3.52 for 6 days and then the ratio fell to an average of 2.6 for 4 days, which is close to the usual Minkowski level. During the first 6 days the increase in protein metabolism above the normal fasting level was only 50 per cent instead of being a threefold increase, which they had usually observed. In 1913 Parhon (4) showed that administration of thyroid preparations with the diet caused little change in muscle glycogen (with thyroid, 0.36 per cent; without thyroid, 0.42 per cent glycogen), whereas such administration caused a profound reaction upon liver glycogen (with thyroid, 0.96 per cent; without thyroid, 5.6 per cent glycogen). Similar results as regards the liver were obtained by Cramer and Krause (5) upon dogs, and Bösl (6) has recently confirmed the work of his predecessors in this field. It appears, therefore, that thyroid secretion tends to release glycogen from its depositories in the liver.

Miura (7), in Fürth's laboratory, administered phlorhizin to fasting cats (1) before removal of the thyroid and parathyroid glands, (2) immediately after this operation, (3) 2 weeks, and (4) about a month thereafter. The D:N urinary ratios in two animals for the 3 day periods were: normal period, 2.68 and 2.79, which were values previously determined by Arteaga (8) as being standard in the cat; after unilateral removal of thyroid and parathyroid, 2.70 and 2.92; after the complete removal of these glands, 2.95 and 3.06; 14 and 17 days after the latter operation, 4.65 and 4.45; and 37 and 29 days after the complete operation, 4.24 and 4.68. In the intervals of time between the different administrations of phlorhizin the cats were well nourished with meat and milk, and hence their body weights were well maintained. After thyroidectomy the nitrogen rose comparatively little in consequence of the administration of phlorhizin. Thus when phlorhizin was given in the normal state the daily nitrogen elimination was 3.5 and 5.2 gm. per day, whereas it was only 1.6 gm. daily on administration of the drug a month after the operation.

Rubner (9), it will be recalled, was the first to associate the increase in protein metabolism of the fasting phlorhizinized dog with the notable increase in the heat production which accompanies this condition.

Falta, Grote, and Staehelin (10) and Murlin and Kramer (11) found that the metabolism of a depancreatized dog rose 42 per cent above the level of the normal basal heat production. Lusk (12) noted that, following the administration of phlorhizin, the metabolism increased in different instances 27, 30, 48, and 70 per cent above the level of the basal metabolism previously measured.

Method of Phlorhizin Administration

To Dog 5, 1 gm. of phlorhizin in 10 cc. of olive oil was given daily subcutaneously. Dogs 67 and 69 received only 0.5 gm. of phlorhizin in the same manner during their diabetic periods.

Lusk's Older Experiments

Thyroidectomy was kindly performed on Dog 5 by Dr. John Rogers, to whom grateful acknowledgment is made. All the thyroid tissue was extirpated and at least one parathyroid was left undisturbed. The operation was begun at 2 p.m. under ether anesthesia, and at 5 p.m. the dog ate with relish the standard diet of this laboratory. This diet consists of meat, 100 gm.; biscuit meal, 100 gm.; lard, 20 gm.; bone ash, 10 gm.; and was given throughout the experiment except when otherwise stated.

Effect of Thyroidectomy on D:N Ratio—The results obtained upon the dog correspond with those obtained by Miura in cats, which latter experiments were published after the results now to be described had been recorded.

The characteristic influence of thyroidectomy on the course of the D:N ratio in phlorhizin glycosuria is best shown in the experiments begun on May 14 about 2 weeks after thyroidectomy and are presented in Period III, Table I.

The standard diet had been administered for 3 days, which allowed for a deposition of glycogen in the tissues. No phlorhizin had been given for 4 days, but the urine contained some glucose at the end of these 4 days. Phlorhizin was again administered about 20 hours after the last meal had been taken. The morning urine of the next day contained 0.128 gm. of nitrogen per hour, the D:N ratio was 8.55, and the "extra sugar," which could not have arisen from protein metabolism, was 0.62 gm. per hour. The morning urine at the end of the 2nd day of diabetes contained 0.101 gm. of nitrogen per hour, the D:N was 9.77, and the extra sugar was again 0.62 gm. On the 3rd morning after the initiation of the diabetes the nitrogen had slightly risen to 0.167 gm., the D:N was 5.42, and the extra sugar 0.30 gm. per hour. The overnight urine, embracing a period of nearly 20 hours, showed a nitrogen content of 0.183 gm. per hour and a D:N ratio of 3.90. During a subsequent period, when muscle tremors were present, the D:N rose to 4.71.

Repetition of this experiment during Period IV, after an interval of 4 days of freedom from phlorhizin and 2 days of administration of the standard diet, gave similar results. The nitrogen in the urine was unaffected and stood at 0.109 gm. per hour, the

TABLE I

Metabolism of Dog 5

Date	Day of test	Day of phlorhizin	Urine analysis: Length of period (min.)	Glucose per hr. (gm.)	N per hr. (gm.)	D:N	Extra glucose per hr. (gm.)	Experiment No.	Length of period (hrs.)	O_2 per hr. (gm.)	r.q.	Non-protein r.q.	Calories per hr.: Protein	Total indirect	Total direct	Body temperature High (°C.)	Low (°C.)	Body weight (kg.)	Remarks
1913																			
Apr. 10	3		190		0.166			1	2	8.85	0.713	0.696	4.40	28.8					Fasting and fever
" 10	3		177	0.885	0.224	3.97		1 a	2	9.09	0.715	0.691	5.94	29.6		39.7	39.1	13.8	Immediately after phlorhizin, 1 gm., subcutaneously
" 11	4	2	248	1.72	0.451	3.81		2	3	12.02	0.684	0.694	5.77	38.4	37.9	40.4	39.9	13.9	Phlorhizin daily to Apr. 12
" 12	5	3	189	1.93	0.505	3.82		3	2	12.15	0.695	0.709	6.46	38.7	36.6	39.3	39.0	13.4	
Apr. 13			188		0.103			4	1	6.88	0.81	0.82	2.73	23.0	19.2	38.9	38.9	11.9	Standard diet daily until May 7
" 29																			Basal metabolism
" 30																			*Thyroidectomy*
May 7			255		0.103			5	2	5.52	0.79	0.79	2.73	18.3				12.1	Basal metabolism
" 8	2	2	249	1.15	0.093	10.89	0.80	6	2	6.24	0.700	0.706	1.23	20.2				11.8	20 hrs. after phlorhizin
" 10	4	4	250	1.20	0.207	5.74	0.45	7	3	6.07	0.696	0.707	2.65	19.4	19.1	38.5	38.2	11.5	3 days " "

																			Notes
May 10-12																			Last phlorhizin May 9. Standard diet
" 13	1	1																	Phlorhizin, 1 gm., 1 p.m.
" 14	2	2	240	1.09	0.128	8.55	0.62												" daily to May
" 15	3	3	240	0.99	0.101	9.77	0.62												16
" 16	4	4	240	0.91	0.167	5.42	0.30												
" 16, 17			1180	0.71	0.183	3.90	0.10												Overnight urine
" 17	5		260	1.09	0.230	4.71	0.25												Tremors. Standard diet 5 p.m.
May 18																			Standard diet 5 p.m.
" 19	1	1																	Skin tough to hypodermic
" 20	2	2	195	1.00	0.112	8.86	0.59	8	1	5.01	0.679	0.683	1.43	16.2	14.7	38.1	37.8	11.0	Pre-calorimeter period
			250	0.85	0.087	9.75	0.53	8a	1	6.05	0.692	0.698	1.43	19.6	18.8	38.1	37.8		Basal. Phlorhizin daily
" 21	3	3	1025	0.81	0.115	7.02	0.39												Tremors
			60	0.94	0.109	8.59	0.54												
			250	1.73	0.447	3.87		9	3	8.33	0.680	0.696	5.70	26.3	22.9	38.9	38.6	10.9	200 gm. meat

D:N was 8.6, and the extra sugar 0.54 gm. at the beginning of the 3rd day of fasting and diabetes.

These persistently high D:N ratios have no recorded parallel in the normal dog after phlorhizinization. One might attribute them to a faculty of converting fat into glucose. However, another explanation seems more reasonable. For in the experiment performed 2 weeks after the extirpation of the thyroid (Period III) 0.62 gm. of extra sugar was eliminated per hour for 48 hours and 0.30 gm. per hour during the next 24 hours, which represents 37.0 gm. of extra sugar eliminated in 3 days from the body of this dog the weight of which was 10.9 kilos. Under quite similar conditions Deuel, Wilson, and Milhorat (13) found that a dog weighing 10.1 kilos, which had been richly fed with carbohydrate 18 hours previous to the administration of phlorhizin, eliminated 33.8 gm. of extra glucose during 26 hours.

Unfortunately, in Lusk's experiments the urine of the early hours after the administration of phlorhizin to the thyroidectomized dog was not collected, but it is quite evident that we are here dealing with a slow mobilization of sugar from the glycogen depositories and a greatly delayed and diminished effect upon the protein metabolism. A further reason why the extra glucose was derived from preformed glycogen and not from fat is based on the respiratory quotient and will be presented under the next heading.

Respiratory Metabolism—Dog 5, prior to the operation and on the 3rd day of fasting (accompanied by fever), eliminated 0.166 gm. of urinary nitrogen per hour (Period I). After 2 days of phlorhizin administration the urinary nitrogen rose to 0.505 gm. per hour, a threefold increase. The D:N of 3.82 was not far from the usual standard of 3.65. The simple fasting metabolism before the injections of phlorhizin showed a heat production of 28.8 calories per hour, with an R.Q. of 0.713, whereas after phlorhizin the heat production rose to 38.7 calories per hour, an increase of 34 per cent. The R.Q. during this diabetic period was 0.695 and the non-protein R.Q. was 0.709, indicating that the non-protein portion of the metabolism was contributed by the oxidation of fat, the R.Q. of which is in the neighborhood of 0.707.

The calculations of the diabetic metabolism are based on the values given by Chambers and Lusk (14) when the D:N ratio is 3.65.

After the dog had received the standard diet for a period of 2 weeks the urinary nitrogen during the morning hours (period of the basal metabolism) was found to be 0.103 gm. per hour (Period II). Thyroidectomy was then performed, the same diet was continued daily, and the "basal nitrogen" on the 8th day following the operation was found to be unchanged. The basal metabolism before thyroidectomy was 23.0 calories per hour, and on the 8th day after the operation 18.3 calories, a fall of 26 per cent.

Phlorhizin was administered and fasting was initiated immediately after this determination of the basal metabolism. 20 hours and again 3 days thereafter the metabolism was determined. In the first experiment the urinary nitrogen was only 0.093 gm., the D:N was 10.89, and the extra sugar eliminated was 0.80 gm. per hour (calorimeter Experiment 6). The total metabolism was 20.2 calories per hour, an increase of only 10 per cent above the normal basal metabolism. After thyroidectomy the non-protein R.Q. was 0.706, the theoretical value for fat. If one assumes that the 0.8 gm. of extra glucose found in the urine of the period arose from fat on the basis of the Bleibtreu (15) formula being reversible,

$$0.37 \text{ gm. fat} + 0.428 \text{ gm. } CO_2 + 0.202 \text{ gm. } H_2O = 1 \text{ gm. glucose}$$
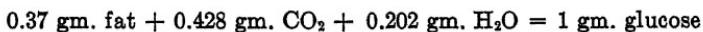
and if one deducts from the 5.36 gm. of non-protein CO_2 found in the respiration of the period, 0.34 gm., which would have been retained if fat were converted into 0.8 gm. of glucose, then one obtains a value for non-protein CO_2 of 5.02 gm. Since the non-protein O_2 was 5.80 gm., a calculated non-protein R.Q. of 0.629 is obtained. Since the theoretical R.Q. for pure fat was in fact observed, it would seem that the extra sugar eliminated could not have had its origin from fat.

Objections may be made that the formula cited is too primitive to warrant consideration. However, the reverse of this formula

$$2.7 \text{ gm. glucose} = 1 \text{ gm. fat} + 1.15 \text{ gm. } CO_2 + 0.55 \text{ gm. } H_2O$$
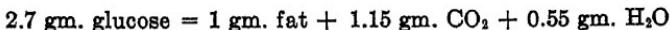
has been successfully employed in this laboratory by Wierzuchowski and Ling (16) in the elucidation of the formation of fat from carbohydrate in the hog.

An alternative method is based on the hypothesis that 3 molecules of palmitic acid are converted into 8 molecules of glucose,

with the accompanying absorption of 21 molecules of O_2. In other words, 0.465 gm. of O_2 would be absorbed when 1 gm. of glucose is produced from fatty acid. Estimating this value for the 0.8 gm. of extra glucose mentioned above, it is found that 0.37 gm. of O_2 must be added to the 5.80 gm. of O_2 appropriated for the oxidation of fat. This would yield an R.Q. of 0.664 instead of one of 0.707 actually found. It follows, therefore, that this extra sugar could not have arisen from fat.

On the morning of the 3rd day after giving phlorhizin the urinary nitrogen had risen to 0.207, the D:N was 5.74, and the extra sugar eliminated was 0.45 gm. per hour (calorimeter Experiment 7). The total metabolism was 19.4 calories per hour, an increase of only 5 per cent above the basal. The non-protein R.Q. was 0.707, or that for pure fat combustion. Had 0.45 gm. of glucose been formed from fat, the O_2 consumption remaining as it was determined, the non-protein R.Q. would have measured 0.680. Here again the evidence is against the supposition that carbohydrate has its origin from fat metabolism.

In a second series of experiments, initiated after 3 days of freedom from phlorhizin injections and 2 days of administration of the standard diet, fasting and phlorhizin injections were again begun (Period IV). 24 hours thereafter the basal metabolism, as measured for 1 hour, was only 16.2 calories, a fall of 11 per cent from the original basal level (calorimeter Experiment 8). In a pre-calorimeter period the nitrogen in the urine was 0.112 gm., the D:N 8.86, the extra sugar 0.59 gm. per hour. During the calorimeter period the urine nitrogen was 0.087 gm., the D:N was 9.75, and the extra sugar was 0.53 gm. per hour. The non-protein R.Q. for the 1st hour was 0.683, which taken by itself would permit the interpretation of a small conversion of fat into glucose. However, in a succeeding hour when manifest tremors in the dog accompanied the experiment, the non-protein R.Q. was 0.698, or very close to that of pure fat. The metabolism rose from 16.2 to 19.6 calories per hour on account of the muscle tremors and this increased heat production was at the expense of fat. Interpretations based on the results obtained in the 1st hour described above are scarcely warranted in view of the other evidence presented.

After administration of 200 gm. of meat to the fasting phlorhizinized thyroidectomized dog the nitrogen in the urine rose from

0.109 gm. per hour on the day previous to 0.447 gm. and the heat production rose from 16.2 calories to 26.3 calories, showing that the specific dynamic action of protein was in full play. The D:N was 3.87 and the non-protein R.Q. was 0.696, indicating that the energy derived from this fraction was essentially from fat. The collection of urine began immediately after the administration of meat. Since the 1st hour of this time was occupied in the preparation of the dog for the calorimeter experiment and since during this hour the urinary nitrogen was scarcely increased, it follows that the average figure of 0.447 gm. of nitrogen given for the calorimeter period is too low. A somewhat greater protein metabolism (with its R.Q. of 0.632 in diabetic condition) would have raised the non-protein R.Q. to a value slightly higher than that calculated, and this would confirm the conclusion that fat is the source of the energy supply of the non-protein quota of the diabetic metabolism.

These experiments lead to the conviction (1) that the increase in heat production which is usually observed in phlorhizin glycosuria does not occur if the thyroid be removed, (2) that the increase in protein metabolism is also less or may be entirely absent, (3) that the loss of extra sugar in the urine as indicated by the D:N ratio is very gradual, (4) that the amount of this extra sugar is within the limits of the power of the body to hold glycogen, and (5) that it is not derived from the transformation of fat into glucose.

The nine non-protein R.Q.'s obtained upon this dog when diabetic, either fasting or fed with meat, averaged 0.697, the highest being 0.709 and the lowest (1 hour only) 0.683.

Newer Experiments

We were handicapped in repeating the experiment just related by the fact that the phlorhizin furnished us by Merck and Company did not possess the same properties as that which was furnished by the same firm 40 years ago. Its administration to dogs rarely produced the D:N ratio of 3.65, but in general one approximating that of Minkowski, 2.8.

Using present day phlorhizin upon the thyroidectomized dog, we have not been able to obtain the high D:N ratios persisting for several days, but we have been able to confirm the fact that there is little or no increase in the basal metabolism after adminis-

Dog 67—This dog was operated on by Dr. J. E. Sweet on November 7, one parathyroid being left *in situ*. Tremors followed by convulsions ensued 5 days later, and were cured by administering 1 cc. of parathyroid hormone (Collip) and 100 cc. of a saturated solution of calcium lactate. Milk instead of meat was given daily with the standard diet. The parathyroid hormone was administered again on November 19 and on December 19. When the experiments reported were begun the dog was receiving the same standard diet as that given to Dog 5 except that it contained 75 instead of 100 gm. of meat. Autopsy revealed parathyroid tissue and a small piece of thyroid tissue about the size of a normal parathyroid gland.

Dog 69—Operation for thyroidectomy was performed by Dr. J. E. Sweet. The thyroid and three parathyroids were removed on January 14. One parathyroid on the left side remained intact. Exploratory operation on April 28 confirmed this condition and the absence of thyroid tissue. Autopsy on May 13 confirmed the findings of the exploratory operation. The same standard diet was received by this dog as by Dog 67.

After meat had been given the nitrogen content of the urine of the dog, obtained during the 5th or 6th hour after meat ingestion, was judged to be a better criterion of the protein metabolism during the hours that the animal was in the calorimeter than the quantity eliminated during the calorimeter period (17). During the early hours after meat ingestion urea gradually increases in the body and the nitrogen in the urine does not accurately reflect the intensity of the protein metabolism of the period. The high D:N ratio of 3.53, found in Dog 67 after 200 gm. of meat were given on February 11 (Table II), does not appear in other similar experiments.

In Dog 67 after thyroidectomy the influence of meat ingestion before and after phlorhizin administration may be thus compared:

Dog 67

Basal metabolism	After meat, 200 gm.	After fasting and phlorhizin administration	
		Basal metabolism	After meat, 200 gm.
calories	*calories*	*calories*	*calories*
11.77	13.93	11.54	13.87
11.78	13.89	11.40	13.37
Average..11.78	13.91	11.47	13.62
Increase over basal metabolism........	2.13		2.15

TABLE II

Metabolism of Dog 6'

Thyroidectomy

Date	Experiment No.	No. of hrs.	O₂ per hr. (gm.)	Urine N per hr. (gm.)	D:N	R.Q.	Non-protein R.Q.	Calories per hr. Protein	Calories per hr. Total indirect	Calories per hr. Total direct	Hrs. after meat	Body temperature High °C.	Body temperature Low °C.	Body weight kg.	Remarks
1929 Nov. 7	6	1	3.54	0.103		0.82	0.83	2.73	11.77	10.12		38.98	38.90	7.0	Basal
1930 Jan. 28	7	3	4.10	0.259		0.87	0.95	6.87	13.93	12.72	3,4,5	39.14	38.69	7.3	Meat, 200 gm.
" 29	8	2	3.49	0.077		0.87	0.88	2.04	11.78	9.56		38.59	38.53	7.3	Basal
" 31	9	2	4.24	0.247		0.84	0.90	6.55	13.89	13.55	3,4	38.80	38.53	7.6	Meat, 200 gm.
Feb. 5		23		0.067											Fasting, 2nd day
" 7		19		0.075	6.39										Fast, 3rd, phlorhizin, 1st day
" 8		28		0.125	3.32										" 4th, " 2nd "
" 9		18		0.165	2.43										" 5th, " 3rd "
" 10	10	3	3.78	0.154	3.25	0.684	0.685	2.35	12.09	11.64				7.0	Basal
" 11		17		0.165	2.67										Fast, 6th, phlorhizin, 4th day
	11	3	3.61	0.160	3.13	0.711	0.722	2.44	11.54	9.19		38.24	38.13	6.8	Basal
	12	2	4.38	0.289	3.53	0.693	0.714	3.70	13.87	13.96	3,5			7.1	Meat, 200 gm.
" 13		39		0.226	2.36										Fast, 8th, phlorhizin, 6th day
		2	3.57	0.167	2.46	0.719	0.733	2.67	11.40	10.41				6.5	Basal
		3	4.27	0.326	2.45	0.707	0.716	5.22	13.37	13.89	3,4,5	38.89	38.78	6.8	Meat, 200 gm.

In this experiment upon Dog 67 there was no increase in the metabolism after giving phlorhizin to the thyroidectomized dog, and the increases after meat was given were exactly the same as similar experiments by Chambers and Lusk (14) have already demonstrated. Of the R.Q.'s obtained with Dog 67 we would question that of 0.719 as being too high for the diabetic condition and out of line with established results.

The results obtained with Dog 69 present the following picture.

Dog 69

Normal		Thyroidectomy		Same, fasting and phlorhizin		Recovery from diabetes	Second period fasting and phlorhizin
Basal metabolism	Meat, 200 gm.	Basal metabolism	Meat, 200 gm.	Basal metabolism	Meat, 200 gm.	Basal metabolism	Basal metabolism
calories	*calories*	*calories*	*calories*	*calories*	*calories*	*calories*	*calories*
13.73	19.62	12.21	16.04	12.40	17.12	13.06	14.16
13.91		11.45	17.23	13.80	16.21		13.73
		11.92	17.14				
Average..13.82	19.62	11.86	16.80	13.10	16.67	13 06	13.95
Increase over basal metabolism........	5.80		4.94		3.57		

It was determined by the work of Chambers and Lusk already cited that the heat production rises to the same height after meat administration to the normal and afterward to the same dog when phlorhizinized. This is reproduced in these experiments for the thyroidectomized dog as well.

These figures might suggest that the specific dynamic action of meat is less after thyroidectomy than in the normal dog and still less in fasting and diabetes. The matter, however, rests largely on one determination of the basal metabolism during fasting and diabetes (13.8 calories per hour), an increase in metabolism not observed in Dog 67.

In a second period of fasting and phlorhizin the metabolism again rose to an average value of 13.95 calories per hour. In virtue of this fact and in view of the variation in the weight of this

dog, it was thought best to calculate the basal metabolism on the basis of the surface area.   It will be remembered that Lusk and Du Bois (18), using the Meeh formula, calculated that the average normal dog produces 772 calories per square meter of surface.

Dog 69

Period	Body weight	Calories, 24 hrs.	Calories, 24 hrs. per sq.m. (Meeh)	Variation from 772 calories per sq. m.
	kg.			*per cent*
1. Normal basal metabolism.......	7.95	332	744	−4
2. After thyroidectomy...........	8.7	285	601	−22
3. Same after phlorhizin adminis-tration......................	8.4	314	679	−12
4. Recovery from diabetes...	8.7	313	662	−14
5. 4th fasting day.................	8.2	308	676	−13
6. After second phlorhizin admin-istration....................	7.3	335	794	+3
7. Depancreatized...............	6.8	306	760	−2

It appears from the accompanying tabulation that the metabolism fell 18 per cent as the result of thyroidectomy.   Though phlorhizin administration caused a rise in metabolism, yet after full recovery from diabetes the metabolism remained unchanged and was also not affected by 3 days of fasting.

On renewed application of phlorhizin, however, the metabolism rose appreciably.   Following the removal of the pancreas the metabolism remained relatively high.   But these are quite different from increases of 42 per cent recorded by Falta, Grote, and Staehelin (10) and by Murlin and Kramer (11), as the result of the removal of the pancreas, or from the usual increase of metabolism in phlorhizin diabetes.   One must therefore conclude with von Noorden that the removal of the thyroid abolishes or greatly diminishes those increases in metabolism which occur when acute diabetes is induced in the dog.

As regards the *protein metabolism* the urinary nitrogen showed average values from 0.096 to 0.106 per hour during Periods 1, 2, 4, 5, and 7.   Only after giving phlorhizin in Periods 3 and 6 did urinary nitrogen rise to 0.179 and 0.166 gm. per hour.   These increases are not as great as those usually found in diabetic animals.

TABLE III

abolism of D₁

Date	Experiment No.	No. of hrs.	O₂ per hr. (gm.)	Urine N per hr. (gm.)	D:N	r.q.	Non-protein r.q.	Protein	Total indirect	Total direct	Hrs. after meat	High (°C.)	Low (°C.)	Body weight (kg.)	Remarks
1929															
Dec. 6	1	3	4.10	0.102		0.85	0.86	2.70	13.73	13.00		38.81	38.79	7.8	Basal
1930															
Jan. 7	2	3	4.13	0.090		0.85	0.86	2.39	13.91	14.21		39.16	38.85	8.1	"
" 10	5	3	6.10	0.437		0.81	0.81	11.58	19.62	17.85	3,4,5	39.08	39.03	8.4	Meat, 200 gm.
" 14															*Thyroidectomy*
Mar. 5	6	1	3.73	0.186		0.82	0.83	4.93	12.21	12.37		37.93	37.79	8.6	Basal
" 12	7	2	3.42	0.103		0.85	0.87	2.73	11.45	11.19		38.10	37.91	8.7	"
" 13	8	3	4.97	0.366		0.83	0.87	9.70	16.04	15.42	3,4,5	37.89	37.75	8.9	Meat, 200 gm.
" 14	9	2	3.56	0.148		0.89	0.94	3.92	11.92	10.67		38.69	38.49	8.8	Basal
	10	3	5.35	0.460		0.84	0.97	12.19	17.23	16.56	3,4,5	38.45	38.03	9.0	Meat, 200 gm.
" 17	11	3	5.24	0.428		0.89	1.09	11.35	17.14	15.87	3,4,5			9.2	" 200
Mar. 20	12	2	3.88	0.155	2.98	0.707	0.714	2.36	12.40	12.34		38.33	37.97	8.4	Fast and phlorhizin, 3rd day. Basal
" 21	13	3	5.53	0.557	2.35	0.725	0.764	8.91	17.12	17.10	3,4,5	39.10	38.87	8.5	Meat, 200 gm.
	14	2	4.34	0.203	2.85	0.695	0.697	3.25	13.80	12.01		38.67	38.49	8.4	Fast and phlorhizin, 4th day. Basal
	15	3	5.25	0.508	2.52	0.697	0.701	8.13	16.21	15.06	3,4,5	39.60	39.08	8.5	Meat, 200 gm.

Date	No.													
Mar. 26	16	3	3.94	0.102		0.82	0.82	2.70	13.06	12.46	38.76	38.41	8.7	Basal
Apr. 8	17	3	3.93	0.088	0.73		0.715	2.12	12.82	13.07	38.35	38.16	8.2	Fast, 4th day
" 12	18	2	4.41	0.179	2.72	0.719	0.725	2.86	14.16	12.78	38.88	38.75	7.6	" 8th, phlorhizin, 3rd day
	19	3	4.26	0.152	2.72	0.726	0.733	2.43	13.73	13.71	39.01	38.84	7.0	" 9th, " 4th "
														Pancreatectomy
May 5	22			0.123	1.65									Fasting
" 9	4		3.93	0.106	2.45	0.715	0.718	1.70	12.73	14.92			6.8	" Basal
	19			0.105	1.90									"

Of the *respiratory quotients* found the non-protein R.Q. of 0.764 (Experiment 13, Table III) after meat is given in diabetes is not in accord with the rest of our experiments and is probably an error. It represents the oxidation of 0.4 gm. of glucose per hour. Excluding this, the average of eleven non-protein R.Q.'s, obtained in Dogs 67 and 69 when phlorhizinized and either fasting or fed with meat, is 0.714, and the variation is betwen 0.685 and 0.733. In Experiment 19 also this non-protein R.Q. of 0.733 is too high for a fasting diabetic dog with a D:N ratio of 2.72. In work with the respiration apparatus all the errors fall upon the O_2 determination, and it is impossible to accept as valid quotients which are out of line with repeatedly established values. If the dog urinates in the calorimeter during the hour the urine is caught in a pan covered with petrolatum, which prevents evaporating urine from occupying air space in the box, thereby apparently raising the R.Q. of the animal. Also it must be realized that even a slight movement by the animal changes the temperature of the surrounding air in a way which is not recorded by the delicate thermometers placed near the wall of the box. Alcohol checks do not participate in all the difficulties encountered when the dog is the subject of experimentation.

The *alcohol checks* covering the period of these observations were made between November 29, 1929, and June 3, 1930. Fourteen checks serially numbered 282 to 295 showed an average value for the R.Q. of 0.664. The maximum value was 0.677, the minimum 0.651. A week before this minimum value was obtained the R.Q. had been 0.667 and 2 days afterward it was 0.665. The average heat as calculated (indirect) was 24.81 calories per hour; the average as measured (direct) was 24.49 calories per hour, a difference of 1.3 per cent. The calorimeter could therefore be depended on.

SUMMARY

1. The increase in heat production which is usually observed after administering phlorhizin to a dog may not occur if the thyroid gland has been removed.

2. The increase in protein metabolism is also less or may be entirely absent.

3. In one dog, in which old-time phlorhizin of Merck was used,

the "extra sugar" was only gradually eliminated from the thyroidectomized animal.

4. The amount of this extra sugar in the urine came within the limits of the power of the body to hold glycogen.

5. That this extra sugar could not have arisen from the metabolism of fat is demonstrated by the respiratory quotients obtained. In this dog, when diabetic, fasting, or fed with meat, nine nonprotein respiratory quotients were obtained which averaged 0.697, the highest being 0.709 and the lowest 0.683.

6. In two thyroidectomized dogs, treated with the modern Merck phlorhizin, the D : N ratios were at the Minkowski level, the delayed elimination of extra glucose was not found, but the other facts given above were confirmed.

BIBLIOGRAPHY

1. Lusk, G., *Proc. XVIIth Internat. Cong. Med.*, London, sect. II, 13 (1913).
2. Minkowski, O., *Verhandl. Kong. inn. Med.*, **14,** 149 (1896).
3. Eppinger, H., Falta, W., and Rudinger, C., *Z. klin. Med.*, **66,** 1 (1908).
4. Parhon, M., *J. physiol. et path. gén.*, **15,** 75 (1913).
5. Cramer, W., and Krause, R. A., *Proc. Roy. Soc. London, Series B,* **86,** 550 (1913).
6. Bösl, O., *Biochem. Z.*, **202,** 299 (1928).
7. Miura, S., *Biochem. Z.*, **51,** 423 (1913).
8. Arteaga, J. F., *Am. J. Physiol.*, **6,** 175 (1901–02).
9. Rubner, M., Die Gesetze des Energieverbrauchs bei der Ernährung, Leipsic and Vienna, 370 (1902).
10. Falta, W., Grote, F., and Staehelin, R., *Beitr. chem. Physiol. u. Path.*, **10,** 199 (1907).
11. Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, **15,** 380 (1913).
12. Lusk, G., *J. Biol. Chem.*, **20,** 555 (1915).
13. Deuel, H. J., Jr., Wilson, H. E. C., and Milhorat, A. T., *J. Biol. Chem.*, **74,** 265 (1927).
14. Chambers, W. H., and Lusk, G., *J. Biol. Chem.*, **85,** 611 (1929–30).
15. Bleibtreu, M., *Arch. ges. Physiol.*, **85,** 345 (1901).
16. Wierzuchowski, M., and Ling, S. M., *J. Biol. Chem.*, **64,** 697 (1925).
17. Lusk, G., The elements of the science of nutrition, Philadelphia and London, 4th edition, 269 (1928).
18. Lusk, G., and Du Bois, E. F., *J. Physiol.*, **59,** 213 (1924).

THE ESTIMATION OF GALACTOSE IN BLOOD AND URINE

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Our knowledge of the metabolism of galactose is hampered by lack of a suitable analytical method. Ordinary bakers' yeast used in comparatively large quantities for a short time has proved a very useful reagent for the removal of glucose from biological fluids such as blood and urine. The difference between the reducing value of a biological fluid, suitably deproteinized or otherwise treated, before and after the use of ordinary yeast constitutes what is often known as "fermentable sugar." Under proper conditions ordinary yeast has no action on galactose, and Corley (1), Blanco (2), and Harding and van Nostrand (3) have been able to gain further information on the metabolism of that sugar by studying the variations in the "fermentable" and "non-fermentable" fractions of blood and urine sugars, if it is assumed that an increase in non-fermentable sugar represents galactose after the ingestion of that sugar. Many yeasts, however, have the power of fermenting galactose, especially if they are first grown on a nutrient medium of hydrolyzed lactose, or on galactose itself. This process of adaptation or acclimatization is well known and was studied in 1908 by Slator (4). Harden and Norris (5) in 1910 showed that the same hexosediphosphate was formed from galactose as was formed from glucose, mannose, and fructose by yeast juice. Both Abderhalden (6) and von Euler and Nilsson (7) showed that galactose-acclimatized yeast could be dried, sterilized, and retain its fermentative power to galactose. There thus seemed no reason why such an acclimatized yeast should not be used as an analytical reagent for galactose, in a manner analogous

to ordinary yeast for glucose. It would first be necessary to remove the ordinary fermentable sugars and then treat the fluid with the acclimatized yeast. The success of such a process would depend largely on the ease with which a galactose-acclimatized yeast could be prepared, and on its selectivity and activity towards galactose under analytical conditions common with, or similar to, those for the removal of glucose.

Preparation of Galactose-Acclimatized Yeast

Ordinary Fleischmann's bakers' yeast is mixed with sterile distilled water, and then centrifuged. The cells are separated from the starch, then washed with sterile distilled water and centrifuged, until the washings are clear. A 50 per cent wet weight suspension is made. The following medium is prepared.

	cc.
Witte's peptone (10 per cent solution)............	20.0
Galactose (20 per cent solution)..................	10.0
$MgSO_4$ (20 per cent solution)...................	0.5
NH_4Cl (20 " " ").....................	1.0
Phosphate mixture* (pH 5.31)......................	10.0
Yeast extract†....................................	40.0

* The composition of the phosphate mixture was KH_2PO_4 60 gm., Na_2HPO_4 6 gm., H_2O 317 gm.

† The yeast extract was prepared by boiling 15 gm. of starch-free yeast 15 minutes with 200 cc. of distilled water, digesting at 90° for 1 hour, and centrifuging till clear and sterilized.

The sterile nutrient medium is placed in a round bottom flask of 150 to 180 cc. capacity and 20 cc. of the yeast suspension are added. The flask is stoppered with a rubber stopper, fitted with a Bunsen valve, or a glass tube ending in a fine capillary. Three or four such flasks are clamped to a small wooden frame, capable of being gently rocked inside an incubator. The mixture is incubated with gentle agitation at 30° for 3 days. The yeast is then separated by centrifugation in sterile tubes, or by decantation, and placed in a similar amount of nutrient medium for a second period of 3 days. This is again repeated. After the third incubation period the final product is freed from reducing substances by washing and centrifuging, and made into a 50 per cent wet weight suspension with water. There is little or no increase in

weight of yeast. One cake of ordinary Fleischmann's yeast produces 9 to 12 gm. of moist weight galactose-acclimatized yeast. It can be kept in the moist state in a refrigerator at 4° for at least a month without losing its galactose-removing properties. The preparation is washed daily with a small amount of distilled water and recentrifuged. For convenience we have called this yeast preparation "galac" yeast.

Activity and Selectivity of Galac Yeast

It is necessary to acclimatize ordinary bakers' yeast to galactose for the full period of three 3 day incubations as described, to obtain a galac yeast of highest activity. The greater part of the acclimatization is accomplished in the first 3 day period. We measured the degree of acclimatization, either by the time of maximal gas production from 100 mg. of galactose, or by the percentage disappearance in reducing power (removal power) of 2 mg. of galactose from 10 cc. of water by 0.5 gm. of wet weight yeast at 38° for 30 minutes. The second and third acclimatization periods result in only a slight difference either in gas production power or removal power. The difference in removal power between the second and third acclimatization periods is distinct, however, and in exact analysis we believe is sufficient to warrant the extra period. Incidentally, we noted that the gas production power and removal power do not run exactly parallel. The disappearance of the galactose as judged by loss in reducing power proceeds faster than the increase in gas production.

The selectivity of galac yeast was examined on a series of sugars of concentrations varying from 2.5 to 20 mg. per cent in aqueous solution; the sugars were treated with the galac yeast under the conditions described under the heading "Estimation of galactose." Galac yeast does not remove maltose,[1] lactose, arabinose, xylose, or glutathione under our analytical conditions. It removes glucose, fructose, mannose, and sucrose as well as galactose. Theoretically it may be expected that galac yeast will possess some removal power on d-talose and d-tagatose, just as ordinary yeast shows an action on mannose and fructose, as well as on glucose.

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[1] We have been informed by other laboratories that ordinary yeast may remove quite appreciable amounts of maltose in 30 minutes. The greatest removal we have seen by galac yeast in 30 minutes is 9 per cent

Estimation of Galactose

Raymond and Blanco (8) showed that 1 cc. of 25 per cent suspension of washed yeast cells removed glucose from 0.2 cc. of blood up to a concentration of 500 mg. per cent in 2 minutes at room temperature. Harding and van Nostrand (3) used the same amount of yeast on 6.0 cc. of Folin-Wu filtrate for a period of 10 minutes at 38°. This has always proved ample to remove glucose from blood or urine when present in the original fluid up to concentrations of 300 mg. per cent. Using this as a basis of comparison, we found that galac yeast was not so active. To be certain that galac yeast removed galactose from blood filtrates and diluted urine when present up to 200 mg. per cent concentration in the original fluid, we found ourselves obliged to increase both the concentration of the moist yeast suspension and the time period.

1.0 cc. of 50 per cent suspension of washed galac yeast is centrifuged, the supernatant liquid poured off, the centrifuge tube dried from adherent moisture by filter paper, and to it are added 10.0 cc. of a galactose solution containing not more than 2 mg. of galactose. The mixture is then incubated 30 minutes at 38° with constant stirring, with either a thin glass rod or stout platinum wire. The mixture is then centrifuged and the supernatant liquid used for analysis. The difference in reducing power of the solution before and after the galac yeast gives the value of the galactose, in absence of the ordinary fermentable sugars. A modified Shaffer-Hartmann reagent was used to determine the reducing power (3). We have applied this to Folin-Wu and Somogyi (Zn) blood filtrates, first removing the "fermentable sugar" by 1.0 cc. of 25 per cent ordinary yeast suspension for 10 minutes at 38° and centrifuging. Urine samples must first be diluted and then cleared by H_2SO_4 and Lloyd's reagent. It is as well to examine closely the supernatant liquid after the use of galac yeast. This yeast does not pack in the centrifuge quite so well as ordinary yeast, and a few cells remaining in the supernatant liquid cause a large increase in the reducing power. In case of any doubt it is advisable to recentrifuge the supernatant liquid. The recovery of galactose added to blood and urine is shown in Table I.

The method of estimation as stated above takes a larger amount

of yeast and a slightly longer time than the usual glucose removal process. We have endeavored to reduce both these factors by experiments on the following lines. (*a*) The use of phosphate mixtures to accelerate the galactose removal; (*b*) preparation of a pure culture from Fleischmann's yeast and acclimatization of the pure culture; (*c*) use of a pure strain of *Saccharomyces validus* grown in a galactose-peptone medium.

TABLE I

Showing Recovery of Added Galactose from Blood and Urine in Fasting

The figures are expressed in mg. per 100 cc. as glucose.

Fluid	Added galactose	Total "sugar"	"Fermentable sugar"	"Galactose sugar"	Residual "sugar"
Blood (dog)	0	106	70	0	36
	100	206	68	102	36
	50	155	69	52	34
	25	125	68	23	34
Urine (1:40 dilution)	0	212	0	0	212
	100	310	0	98	212
Urine (1:20 dilution)	100	345	0	97	248
Blood (rabbit)	0	116	86	0	30
	100	214	84	99	31
	50	165	83	51	31
Urine (human) (1:10 dilution)	0	57	6	0	51
	100	158	6	100	52
	50	111	7	52	52
	25	85	9	26	50

The following is a brief summary of the results. (*a*) 0.6 per cent KH_2PO_4 solution used as a solvent for galactose instead of water slightly accelerated the removal of galactose in higher concentrations. Over 15 minutes, however, were still required to remove completely 2 mg. of galactose in 10 cc. of solution. (*b*) The pure culture from Fleischmann's yeast gave the same results as the bought material on acclimatization. (*c*) *Saccharomyces validus* is superior to galac yeast as a galactose remover, 2 mg. of galactose in 10 cc. disappearing in 20 minutes. We doubt, however, if the

difference is sufficient to compensate for the trouble of keeping a pure strain of yeast in the ordinary biochemical or physiological laboratory, when the standard Fleischmann's yeast is so readily obtainable. Other varieties of yeast, however, are known to possess higher galactose-fermenting powers than those we examined.

Examination of Normal Blood and Urine for Galactose

In Table I, showing the recovery of galactose added to blood and urine, we have given the amounts present in the fasting condition as zero. The actual presence of very small amounts of galactose in blood and urine is, however, of importance. A few workers (9) have raised the question of another true sugar in blood. We have, in consequence, submitted blood and urine during fasting to a careful examination for galactose. Previous experiments had shown us that a single treatment of Folin-Wu blood filtrate by ordinary washed yeast sometimes left behind a small amount of "fermentable sugar." These occasional amounts varied around 2 mg. per 100 cc. of blood. If under these conditions the filtrate was next treated with galac yeast, this small amount of sugar was removed and appeared in the analysis as galactose. The failure to remove the last traces of fermentable sugar is probably due to an insufficient contact between the cells and the liquid. Continuous stirring removes this source of error.

We examined plasma and corpuscles separately for the presence of galactose by the following special technique.

(1) *Fermentable Sugar Removal*—12.0 cc. of Folin-Wu filtrate (plasma 1:5, corpuscles 1:10) are added to 0.50 gm. of washed ordinary yeast in a centrifuge tube and warmed in a bath at 38° for 10 minutes with continuous stirring. The yeast is removed by centrifugation. The supernatant liquid is again centrifuged to remove any possible stray yeast cells. Two 2.0 cc. portions are removed for analysis. On the remaining liquid the yeast treatment is repeated, 0.25 gm. of yeast (ordinary) being used, and the supernatant liquid analyzed for reducing substances after double centrifuging. The second treatment with yeast should yield no further fermentable sugar if the first has been efficient. We have found no difference between the two treatments.

(2) *Fermentable Sugar Plus Galactose Removal*—A second 12.0 cc. of the same Folin-Wu blood filtrate are treated with 1.0 gm. of

moist washed galac yeast at 38° for 30 minutes with constant stirring. Double centrifuging of the liquid is again resorted to. After samples are withdrawn for analysis the galac yeast treatment is repeated (0.50 gm. of galac yeast being used). Under these conditions we found the removal of sugars by the galac yeast complete after the first treatment.

TABLE II

Illustrating Removal Action of Ordinary Yeast and Galac Yeast on Folin-Wu Blood Filtrates, and Absence of Galactose

The figures are expressed in mg. per cent as glucose.

Normal male blood	Total reduction	Residual reduction				Galactose sugar $(a) - (b)$
		After ordinary yeast (a)		After galac yeast (b)		
		1st treatment	2nd treatment	1st treatment	2nd treatment	
Plasma......................	119	11	10	9	9.5	+1
Corpuscles.................	109	50	50	49	50	±0
Plasma......................	110	8.5	8.5	8	8	+0.5
Corpuscles.................	102	41.5	41.5	41.5	41	±0
Plasma......................	108	7	6	6	5.5	+1
Corpuscles.................	102	43.5	42	42	43	±0
Plasma..	107	8	8	8	8	±0
Corpuscles.................	109	48	46	46	48	±0
Plasma......................	115	10.5	11	10.5	11.5	±0
Corpuscles.................	120	60.5	62	61.5		±0

The difference represents galactose. The results on normal blood of fasting males are illustrated in Table II. The procedures described under (1) and (2) were also applied to normal urine in fasting after dilution and treatment with H_2SO_4 and Lloyd's reagent.

Within the error of experiment no galactose was found. The occasional +1 mg. of galactose reported is well within our error of experiment. The negative results on normal urine in fasting are shown in Table III.

TABLE III

Showing Absence of Galactose from Urine of Non-Pregnant, Pregnant, and Puerperal Individuals During Fasting and Presence of "Fermentable" and "Galactose" Sugar on Hydrolysis

The figures are expressed in mg. per 2 hours.

Subject	Benedict test	Before hydrolysis			After hydrolysis		
		"Fermentable sugar"	"Galactose sugar"	Residual reduction	"Fermentable sugar"	"Galactose sugar"	Residual reduction
Female (non-pregnant)	Negative	0	0	24	17	6	7
Male.................	"	0	0	43	21	5?	52
"	"	7	0	49	23	4?	46
"	"	0	0	40			
"	"	0	0	65			
"	"	0	0	29			
"	"	0	0	45			
Antepartum..........	"	0	0	20			
"	"	5	0	92	45	28	67
"	"	0	0	113	40	38	92
"	"	0	0	118	43	37	54
"	"	0	0	86	51 ·	20	74
"	"	0	0	78	28	20	77
"	"	0	0	32	12	10	38
"	Positive	0	0	245	172	69	176
Postpartum...........	Negative	0	0	99			
"	Positive	0	0	1620	1157	623	1050
"	"	87	0	478	327	163	277
"	"	0	0	137	88	61	128
"	Negative	0	0	122	65	29	94
"	"	0	0	117	73	46	84
"	"	9	0	103	62	28	90
"	"	5	0	38	18	10	40
"	Positive	0	0	392	242	131	209

Examination of Blood and Urine in Pregnancy for "Galactose Sugar"

We similarly examined four blood and urine samples of fasting patients in the last month of pregnancy and six blood and urine samples 3 to 4 days post partum. The collections were made in the Burnside Metabolism Ward of the Toronto General Hospital by

a special nurse.   Catheter collection was employed for the post-partum urine samples.

The results were exactly similar to those obtained on the normal male blood and urine.   We thus have been unable to demonstrate the presence of circulating or excreted galactose, although in the postpartum cases lactation had commenced and the urine often contained considerable amounts of non-fermentable reducing substances (Table III).

Examination of Hydrolyzed Urine from Pregnant and Puerperal Women for "Fermentable Sugar" and "Galactose Sugar"

It is well known that the urine passed in pregnancy and after labor often shows strong reducing properties in absence of any recognizable amount of glucose.   It is customary to refer to such a condition as a lactosuria.   Watkins (10) has recently reviewed the evidence on which such a conclusion is based and believes that the designation of lactosuria is correct.   More recently in a preliminary note Winter (11) has stated himself unable to isolate d-phenyllactosazone from such urine samples, although he was able to recover lactose added to other urine samples in quantities similar to those reported by analysis.   His examination of the literature led him to the conclusion that there existed no real proof of the presence of lactose in urine in pregnancy.   He isolated lactose, however, from the urine of lactating women.

A simple extension of the action of ordinary and galac yeast results in a method for the examination of urine for lactose.   The lactose is hydrolyzed by 0.5 N H_2SO_4, the acid removed by solid $BaCO_3$, the solution filtered, and analyzed successively for glucose and galactose.   Preliminary tests were made as follows:

25.0 cc. of 0.02 per cent lactose solution are mixed with 25.0 cc. of N H_2SO_4 in a pressure bottle and heated 1 hour in a boiling water bath.   While still hot, the solution is neutralized with solid $BaCO_3$ and filtered.   A portion of the filtrate is used for analysis. The amounts of glucose and galactose obtained are not theoretical. Under our conditions of hydrolysis, however, the glucose and galactose are produced in approximately equimolecular amounts. More complete hydrolysis can be obtained by adding an equal volume of 2 N H_2SO_4 and heating for 1 hour.   The proportion of glucose and galactose produced by the stronger acid as determined

by yeast analyses, however, is altered from the expected 1:1 ratio. We feel, however, it is preferable to use the weaker acid and if the lactose percentage is desired to calculate it as hydrate from the amount of galactose, on the basis of 72 per cent hydrolysis. Lactose added to normal urine of fasting males or females gives similar recovery figures (Table IV). We have applied this method of hydrolysis to urine of pregnant and puerperal women (Table III). The urine samples often showed a strongly positive Benedict test, and containing no glucose, would be designated as lactosurias. The amount of non-fermentable reducing material in these urine samples is sometimes considerable. On hydrolysis both "fermentable sugar" and "galactose sugar" are easily demonstrable.

TABLE IV

Showing Hydrolysis of Lactose in Water and Urine and Production of Glucose and Galactose in Equimolecular Amounts

Added lactose	Recovered sugar		Hydrolysis of lactose
	Glucose	Galactose	
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*
200 mg. in 100 cc. water...........	74	72	73
100 " " 100 " "	37	37	74
80 " " 100 " urine...........	30*	25	70

* Corrected for the "fermentable sugar" produced by hydrolysis from the urine without added lactose.

If the lactosurias of pregnancy are produced by the simple absorption of lactose from the mammary gland and its excretion in the urine, it would be expected that the amount of "fermentable sugar" and "galactose sugar" produced by our method of hydrolysis would approach the molecular ratio of 1:1 in those urines showing a large amount of non-fermentable sugar. Our figures show a ratio of more nearly 2:1 in such urines and thus do not correspond with the idea that the lactosurias of pregnancy and the puerperium are produced by the simple addition of lactose to urine.

On the other hand urine both from cases of pregnancy and puerperium shows amounts of "galactose sugar" after hydrolysis in considerable excess of those found in the three samples of urine from a non-pregnant fasting individual.

SUMMARY

Ordinary bakers' yeast grown in a medium containing galactose can be utilized to remove galactose quantitatively from aqueous solutions, from Folin-Wu blood filtrates, and from urine treated with H_2SO_4 and Lloyd's reagent.

No galactose can be found in normal blood in fasting, either plasma or corpuscles, nor in normal urine in fasting.

No galactose can be found in fasting blood or urine, in late pregnancy, or 3 days post partum at the beginning of lactation.

The non-fermentable "sugar" found in fasting urine, in late pregnancy, and the puerperium on hydrolysis gives "fermentable sugar" and "galactose sugar." The amounts of "fermentable sugar" and "galactose sugar" on hydrolysis are inconsistent with the idea that lactosurias are produced by the simple addition of lactose to the normally occurring non-fermentable urinary reducing substances.

BIBLIOGRAPHY

1. Corley, R. C., *J. Biol. Chem.*, **74,** 1 (1927).
2. Blanco, J. G., *J. Biol. Chem.*, **79,** 667 (1928).
3. Harding, V. J., and van Nostrand, F. H., *J. Biol. Chem.*, **85, 765** (1929–30).
4. Slator, A., *J. Chem. Soc.*, **93,** 217 (1908).
5. Harden, A., and Norris, R., *Proc. Roy. Soc. London, Series B,* **82, 645** (1910).
6. Abderhalden, E., *Fermentforschung,* **8,** 42 (1924).
7. von Euler, H., and Nilsson, R., *Z. physiol. Chem.*, **143,** 89 (1925).
8. Raymond, A. L., and Blanco, J. G., *J. Biol. Chem.*, **79,** 649 (1928).
9. Folin, O., and Malmros, H., *J. Biol. Chem.*, **83,** 121 (1929). Hubbard. R. S., and Deegan, J. K., *J. Biol. Chem.*, **86,** 577 (1930).
10. Watkins, O., *J. Biol. Chem.*, **80,** 33 (1928).
11. Winter, L. B., *J. Physiol.*, **69,** p. viii (1930).

THE ALKALINE DEAMINATION OF DERIVATIVES OF CYSTEINE*

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In the course of a study of the action of alkali upon cystine and cysteine (4) it was shown that cysteine is far more stable towards boiling alkaline reagents than is cystine under the same conditions. A possible explanation for this difference may be found in the attachment to the sulfur atom of the weakly polar hydrogen atom, in contrast to the highly unsaturated sulfur atom in the disulfide. In order to test this view, the rates of deamination of the S-ethyl, S-benzyl, and S-phenyl derivatives of cysteine have been determined under various comparable conditions. Curves indicating the results of these experiments are shown in Figs. 1 to 4.

The three cysteine derivatives exhibit the same general progress of deamination when treated with the respective reagents, the reactivity being in the general order: ethyl < benzyl < phenyl. Cystine, in harmony with the observations of Baumann (1), Brenzinger (3), and Friedmann (6), takes a position intermediate between the benzyl and the phenyl derivatives. In the decomposition of all of these compounds with suspensions of lime and with alkaline plumbite, the autocatalytic type of reaction curve, discussed in our previous communication, is clearly seen. That the active agent in this process is pyruvic acid is demonstrated by the abolition of the induction period on the addition of salicylaldehyde, which acts similarly to pyruvic acid but differs from it in being relatively stable towards alkali.

In view of the work of Baumann and Preusse (2), Brenzinger

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† Died September 17, 1931.

(3), and Friedmann (6) on bromophenylcysteine and ethylcysteine, it was considered unnecessary rigorously to establish the formation of pyruvic acid during the decomposition of phenylcysteine. In all of the experiments involving salicylaldehyde the typical orange coloration characteristic of its condensation product with pyruvic acid was developed.

It is of interest to note that while the evolution of ammonia from cystine in N sodium hydroxide alone ceases when about one-
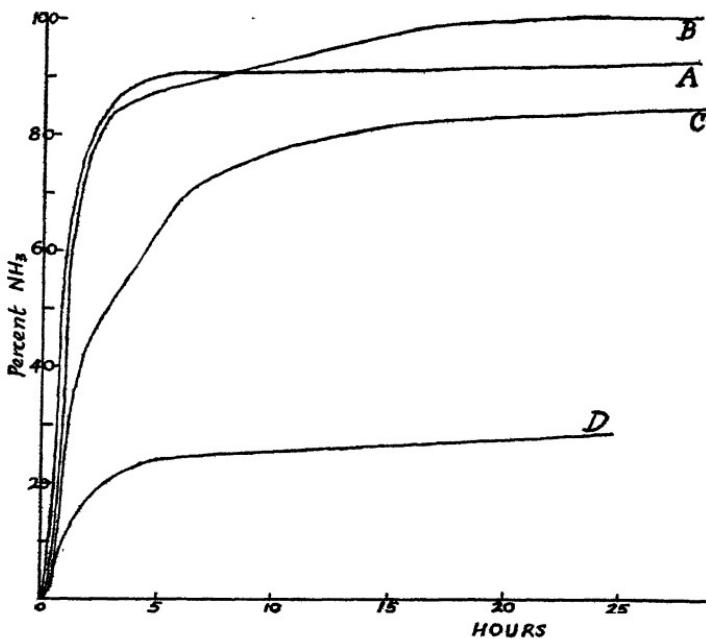
FIG. 1. Cystine. Curve A, cystine + salicylaldehyde + NaOH; Curve B, cystine (as Na salt) + Ca(OH)₂; Curve C, cystine + PbO + NaOH; Curve D, cystine + NaOH.

third of the theoretical amount has been evolved, this is not true of the derivatives of cysteine, which appear to approach complete deamination, just as is the case with cystine when subjected to the action of lime, alkaline plumbite, or alkaline salicylaldehyde.

Since the effect of sodium p-hydrazinobenzoate on the rate of deamination in N sodium hydroxide is but slight, it seems reasonable to infer that in boiling alkali only small amounts of un-
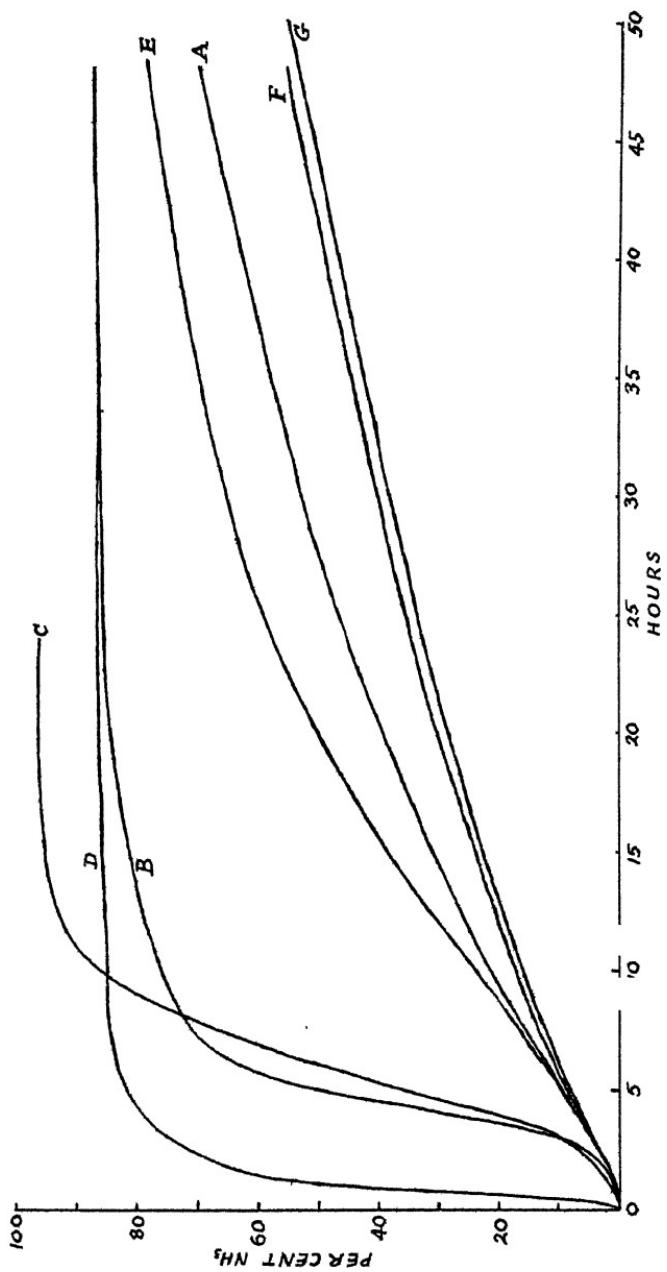
FIG. 2. S-Ethylcysteine. Cu ve A, ethylcysteine + NaOH; Curve B, ethylcysteine + NaOH; Curve C, ethylcysteine + Ca(OH)₂; Curve C, ethylcysteine + NaOH + PbO; Cu re D, ethylcysteine + salicylaldehyde +NaOH; Curve E, ethylcysteine + NaOH + Ca(OH)₂ Curve F, ethylcy eine + Na hydrazinobenzoate + NaOH; Curve G, ethylcysteine + Na hydrazinobenzoate + NaOH + PbO.

changed pyruvate are present at any instant. While the substitution of lime for sodium hydroxide always greatly increases the rate of deamination, addition of lime to the alkali has only a relatively small effect. This may be explained by attributing to the lime a milder condensing ability upon pyruvic acid, as well as by the lower solubility of calcium hydroxide in N alkali.

The interpretation of the behavior of lead oxide offers considerable difficulty. As in the case of cystine, the presence of plumbite
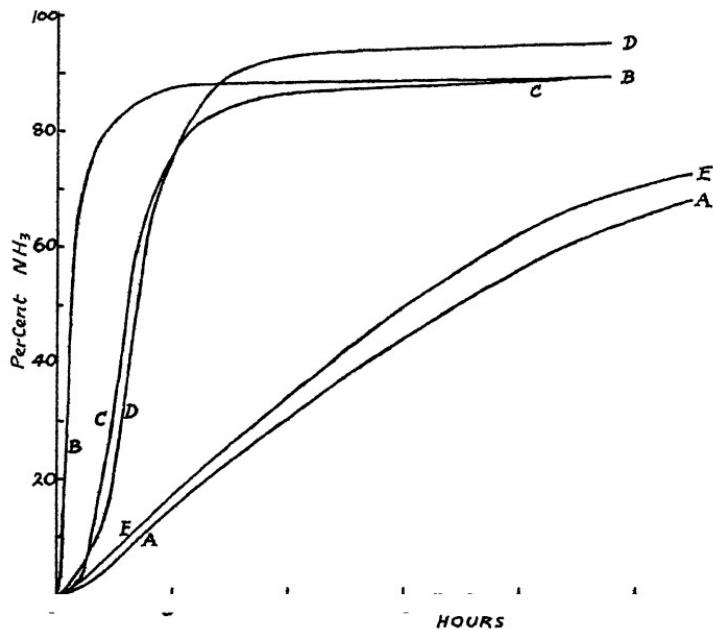
FIG. 3. S-Benzylcysteine. Curve A, benzylcysteine + NaOH; Curve B, benzylcysteine + NaOH + salicylaldehyde; Curve C, benzylcysteine + Ca(OH)$_2$; Curve D, benzylcysteine + NaOH + PbO; Curve E, benzylcysteine + NaOH + Ca(OH)$_2$.

in the alkali markedly increases the rate of deamination. This effect, however, is completely nullified by the introduction of the hydrazine derivative.

The absence of effect of plumbite is also seen in the experiments with cysteic acid (Fig. 5). This compound, in which the sulfur exists in its fully oxidized condition, is extremely stable towards boiling alkali. Indeed, Friedmann (6) stated that ammonia is

A-Phenylcysteine + NaOH
B-Phenylcysteine + Ca(OH)₂
C-Phenylcysteine + NaOH + PbO
D-Phenylcysteine + Salicylald. + NaOH
E-Phenylcysteine + NaOH + Hydroazinobenzoate
F-Phenylcysteine + NaOH + PbO + Hydroazinobenzoate
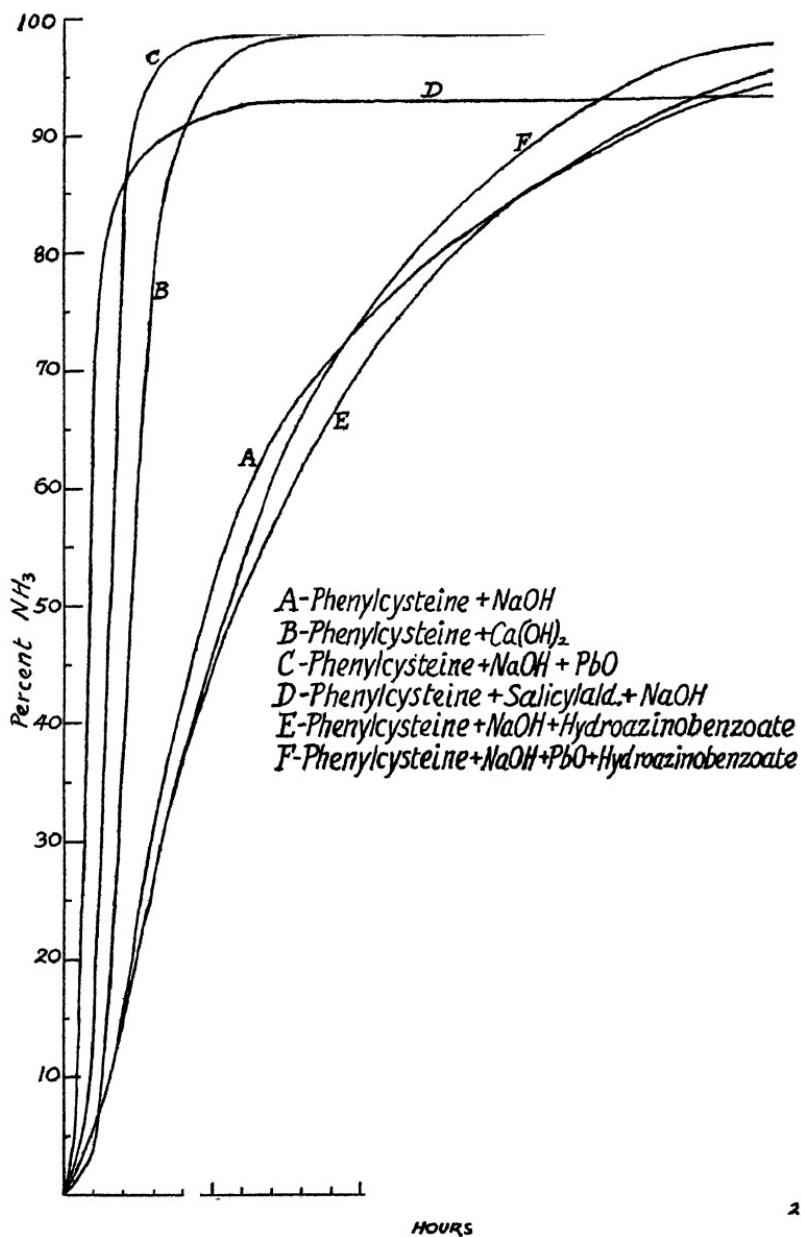
FIG. 4. S-Phenylcysteine

evolved on heating with alkalies only to temperatures above 100°. We find that on treatment with N sodium hydroxide at 100° about 2 per cent of the nitrogen is split off as ammonia during 96 hours;
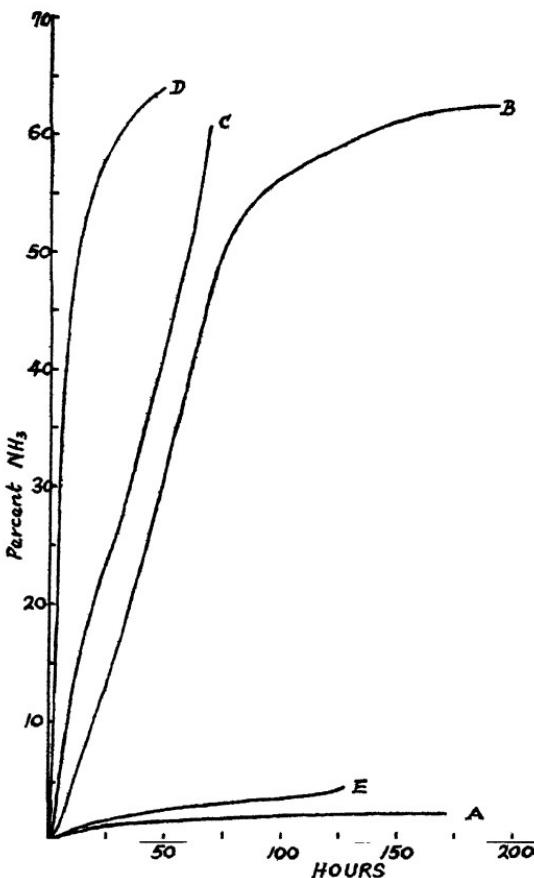
FIG. 5. Cysteic acid.   Curve A, cysteic acid + NaOH; Curve B, cysteic acid + Ca(OH)₂; Curve C, cysteic acid + NaOH + salicylaldehyde; Curve D, cysteic acid + Ca(OH)₂ + salicylaldehyde; Curve E, cysteic acid + NaOH + PbO.

with a 5 per cent suspension of lime under similar conditions, 50 per cent of the nitrogen appears as ammonia during about 80 hours, while the addition of salicylaldehyde causes an even greater increase in reaction rate.

As in the cases above discussed, the formation of an orange color during the decomposition of cysteic acid in the presence of salicylaldehyde points to the formation of pyruvic acid, which has also recently been demonstrated by Daft and Coghill (5) in the case of serine. The sulfur is split out almost entirely in the form of sulfites.[1] Thus, in an experiment with lime and water at 100°, in which the decomposition was interrupted after the expulsion of 62 per cent of the theoretical amount of ammonia, 40.9 per cent of the sulfur originally present was recovered as sulfurous acid and 0.47 per cent as sulfuric acid. In a similar experiment with N sodium hydroxide, 2.44 per cent of the nitrogen was recovered as ammonia after 168 hours, 0.56 per cent of the sulfur appearing as sulfurous acid.

In another experiment in which a 0.05 M solution of cysteic acid in a 5 per cent suspension of lime was boiled over a free flame in a current of hydrogen, the amount of ammonia recovered after 51 hours was 62.4 per cent of the theoretical value, while 70.9 per cent of the sulfur originally present was recovered as sulfite and 8.0 per cent as sulfate. The reaction which occurs on heating cysteic acid with alkaline solutions accordingly appears to be best expressed by

$$\begin{array}{c} CH_2SO_3H \\ | \\ CHNH_2 \\ | \\ CO_2H \end{array} \xrightarrow{\ NaOH\ } \begin{array}{c} CH_3 \\ | \\ CO \\ | \\ CO_2Na \end{array} + NH_3 + Na_2SO_3$$
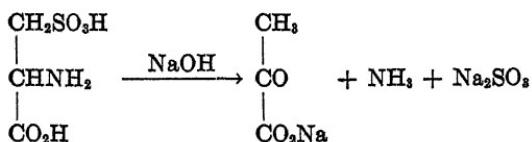
EXPERIMENTAL

As in previous experiments (4), the evolved ammonia was estimated by aspiration through a reflux condenser with a gentle current of moist hydrogen. Owing to the bumping experienced with actively boiling solutions, the present series was heated by a boiling water bath instead of a free flame. The cysteine derivatives were treated in 0.05 M solutions, the cystine in 0.025 M. During the alkaline decomposition of the alkyl cysteines the odor of mercaptans soon became evident, except when plumbite was present. In the latter case, yellow lead mercaptides separated.

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[1] Friedmann (6) reported the formation of considerable amounts of barium sulfite on heating a solution of the barium salt under pressure.

S-Ethylcysteine—This compound has hitherto been prepared (3) only by the action of ethyl iodide upon the mercuric chloride derivative of cysteine. The procedure here adopted furnishes considerably better yields. To a solution of 24 gm. of cystine (having $[\alpha]_{546}^{23.5} = -247°$, 4 per cent in N HCl) in 200 cc. of 10 per cent hydrochloric acid are added 16 gm. of metallic tin; the mixture is gently boiled for 2 hours, cooled, and decanted from a little unchanged tin into a mixture of 55 gm. of sodium hydroxide, 75 cc. of water, and 400 cc. of 95 per cent ethyl alcohol. The resulting solution is cooled to 25° and treated with 46 gm. of ethyl sulfate. The temperature rises to 35° and then gradually falls. After 3 hours the mixture is acidified with 40 cc. of glacial acetic acid and freed of alcohol by distillation under reduced pressure. It is then acidified to Congo red with hydrochloric acid, saturated with hydrogen sulfide, and freed of tin sulfide by filtration. The filtrate is concentrated under reduced pressure until sodium chloride separates; an equal volume of alcohol is added, and the precipitate of sodium salts is filtered off and washed with 50 per cent alcohol. Filtrate and washings are again concentrated, and the process repeated. The filtrate is freed of alcohol as before, diluted with about twice its volume of water, boiled for half an hour to hydrolyze any ester, and treated hot with a slight excess of ammonia, when ethylcysteine crystallizes almost immediately. When cold, the mixture is filtered by suction and the product washed free of salts with cold water. A second crop is obtained by evaporating the mother liquor and washings under reduced pressure. In this way 22.5 gm. (75 per cent of the theoretical amount) of S-ethylcysteine can readily be obtained. The product crystallizes from 50 per cent alcohol in colorless leaflets melting at about 260° (corrected) and yielding on analysis satisfactory values for nitrogen and sulfur.

The following polarimetric values were obtained.

$$[\alpha]_{546}^{23} = -27.4°, \ [\alpha]_{D}^{25} = -23.7° \ (3.2 \text{ per cent in water})$$
$$[\alpha]_{546}^{24} = -3.8° \ (2.8 \text{ per cent in N HCl})$$
$$[\alpha]_{546}^{24} = +5.2° \ (3.3 \ " \ " \ " \ " \text{ NaOH})$$

S-Benzylcysteine—This was prepared in the same way as the ethyl derivative, the ethyl sulfate being replaced by 38 gm. of

benzyl chloride; a 62 per cent yield of recrystallized product (m.p. 215–216° (corrected) with decomposition) was secured, possessing the properties recorded in the literature (7, 8).

$$[\alpha]^{23}_{546} = -0.3° \quad (3 \text{ per cent in n HCl})$$
$$[\alpha]^{24}_{546} = +22.7° \quad (3.2 \text{ " " " " NaOH})$$

S-Phenylcysteine—This compound has hitherto only been obtained by reduction of its *p*-bromo derivative secured from bromophenylmercapturic acid (1). Its synthesis (in about 30 per cent yield) was accomplished in the following manner. A solution of cysteine hydrochloride obtained from 12 gm. of cystine by reduction with tin in hydrochloric acid, followed by the removal of the tin as the sulfide, is neutralized with ammonia and treated with a neutralized diazobenzene solution from 10 gm. of aniline, the temperature being held below 5° during the addition. Evolution of gas occurs at once, and a small quantity of a flocculent yellow precipitate forms. The mixture is allowed to warm up to room temperature and to stand overnight, whereupon it is filtered. The filtrate is acidified with acetic acid, concentrated to about 300 cc., and allowed to stand overnight at room temperature. The crude product which separates is filtered off, dissolved in dilute hydrochloric acid, decolorized with charcoal, and reprecipitated with sodium acetate. It now weighs about 8 gm. and consists of a mixture of phenylcysteine and cystine. The former is isolated by extraction with hot 50 per cent alcohol (in which the cystine is sparingly soluble) and repeatedly recrystallizing from the same solvent. The phenylcysteine so prepared forms flat needles melting at 201–202° (corrected) with evolution of gas.

Analysis

3.885 mg. gave 2.03 mg. H_2O, 7.845 mg. CO_2. H, 5.84; C, 55.07.
3.873 " " 2.02 " " 7.835 " " " 5.83; " 55.10.
4.303 " " 0.268 cc. moist nitrogen (23°, 752 mm.). N, 7.11.
7.950 " " 9.32 mg. $BaSO_4$. S, 16.10.

$C_9H_{11}O_2NS$ requires C, 54.82; H, 5.58; N, 7.11; S, 16.25

Rotation

$$[\alpha]^{23.5}_{546} = +82° \quad (6.2 \text{ per cent in n HCl})$$
$$[\alpha]^{22.5}_{546} = +13.4° \quad (2 \text{ " " " " NaOH})$$

Cysteic Acid—This was prepared by a modification of the method of Friedmann (6). To a cold solution of 24 gm. of cystine in 50 cc. of concentrated hydrochloric acid diluted with 150 cc. of water 80 gm. of bromine are added, with occasional stirring, during 40 minutes. The temperature rises to about 60°. The resulting solution is evaporated to dryness on the steam bath under reduced pressure, and the residue is recrystallized from water. A yield amounting to nearly 90 per cent of the theoretical quantity of cysteic acid monohydrate is obtainable when care is taken to recover the product contained in the mother liquors. Cysteic acid monohydrate forms colorless needles which melt with decomposition at 289° (corrected).

Rotation

$$[\alpha]_{546}^{24.5} = +9.4° \quad (6 \quad \text{per cent in water})$$
$$[\alpha]_{546}^{27} = +11.8° \; (2.3 \quad `` \quad `` \quad `` \quad \text{N HCl})$$
$$[\alpha]_{546}^{27} = +1.0° \; (2.4 \quad `` \quad `` \quad `` \quad `` \quad \text{NaOH})$$

SUMMARY

1. The alkaline deamination of S-substituted cysteines proceeds along the same general lines as those followed under the same conditions by cystine and cysteine.

2. The stability of these compounds towards alkali is largely influenced by the degree of unsaturation of the group attached to the sulfur atom. This affords an explanation of the increase of reactivity displayed by cystine as compared to cysteine.

3. Cysteic acid is decomposed by alkaline reagents in an analogous manner, but with a lower order of velocity than with cysteine and its derivatives.

BIBLIOGRAPHY

1. Baumann, E., *Ber. chem. Ges.*, **15**, 1731 (1882).
2. Baumann, E., and Preusse, C., *Z. physiol. Chem.*, **5**, 309 (1881).
3. Brenzinger, K., *Z. physiol. Chem.*, **16**, 552 (1892).
4. Clarke, H. T., and Inouye, J. M., *J. Biol. Chem.*, **89**, 399 (1930).
5. Daft, F. S., and Coghill, R. D., *J. Biol. Chem.*, **90**, 341 (1931).
6. Friedmann, E., *Beitr. chem. Physiol. u. Path.*, **3**, 1 (1903).
7. Suter, F., *Z. physiol. Chem.*, **20**, 562 (1895).
8. du Vigneaud, V., Audrieth, L. F., and Loring, H. S., *J. Am. Chem. Soc.*, **52**, 4500 (1930).

THE DISTRIBUTION OF PHOSPHATASE IN THE TISSUES OF TELEOSTS AND ELASMOBRANCHS

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The rôle of the phosphatases in ossifying processes has attracted increasing attention in recent years, chiefly from two points of view. First, Robison and his coworkers (1) have considered the presence of phosphatase in bone and ossifying cartilage as an agent active in ossification. Secondly, the relation between equilibria involving bone deposition or resorption and the phosphatase content of other tissues or of blood has been investigated. Thus Kay (2) has reported increases in plasma phosphatase in various bone diseases. Heymann (3) noted a decreased phosphatase content of the upper intestine in experimental rickets. Page and Reside (4) found decreases in the phosphatase content of intestinal mucosa and kidney after administration of vigantol, and Page (5) reported a diminished bone phosphatase activity after parathormone injection.

The purpose of this investigation was to study the distribution of phosphatase in the tissues of bony and cartilaginous fish, vertebrates which have a different type of skeletal tissue from the mammals.

Procedure

The experiments were performed during the months of July and August. The smallest obtainable and presumably therefore the younger individuals of each species were used. Except in the case of the sharks and one skate, *Raia erinacea* 2, the organs were

removed immediately after death; in the exceptions mentioned, the tissues were obtained within 2 hours after death.

The common and scientific names of the species employed are given below. The sex and the weight in gm. or, in the case of the sharks and the torpedo ray, the length in feet are indicated for the various individuals.

Elasmobranchs

Smooth dogfish, *Mustelus canis*, No. 2, 485 gm.; No. 3, 537 gm.; No. 4, 1160 gm.; No. 5, 365 gm.; No. 6 ♂, 136 gm.; No. 7 ♂, 146 gm.; No. 8 ♂, 434 gm.; No. 9 ♀, 580 gm.

Common skate, *Raia erinacea*, No. 1 ♀, 474 gm.; No. 2 ♀, 635 gm.

Barndoor skate, *Raia stabuliforis* 1 ♂, 2100 gm.

Mackerel shark, *Isurus nasus* 1 ♀, about 3 feet.

Dusky shark, *Carcharhinus obscurus*, No. 1 ♂, about 4 feet; No. 2 ♂, about 4 feet.

Torpedo ray, *Narcacion nobiliana* 1 ♀, about 2.5 feet.

Teleosts

Butterfish, *Rhombus tricanthus*, No. 1, 135 gm.; Nos. 2 and 3 pooled, each about 160 gm.; No. 4 ♂, 270 gm.

Common mackerel, *Scomber scombrus*, No. 1, 170 gm.; Nos. 2 and 3 pooled, both ♀, each about 100 gm.; No. 4 ♀, 191 gm.

Scup, *Stenotomus chrysops*, No. 1, 312 gm.; No. 2 ♂; Nos. 3 and 4 pooled, each about 70 gm.

Weakfish, *Cynoscion regalis* 1 ♂, 665 gm.

Sea herring, *Clupea harengus*, No. 1, 190 gm.; No. 2 ♂, 145 gm.; Nos. 3 and 4 pooled, both ♀, each about 210 gm.

Tautog, *Tautoga onitis*, No. 1 ♂, 341 gm.; No. 2 ♀, 356 gm.; No. 3 ♀, 205 gm.

Comon puffer, *Spheroides maculatus*, No. 1 ♂, 160 gm.; No. 2.

In general, the procedure of Kay (6) for the extraction of mammalian phosphatases was followed. The tissues were weighed moist with an error no greater than 2 to 3 per cent, and washed with several volumes of 0.65 per cent saline. The skeletal tissue consisted of one or more vertebræ as free as possible of muscle and nerve. Since the gastrointestinal tract was usually small, the entire wall, rather than the mucosa, was used. The tract was opened and divided into the various portions before washing. In many instances, the washings were saved and tested for phosphatase activity.

The tissue was cut, then ground with 1 to 2 gm. of washed and

ignited sea sand, glass often being added to the skeletal tissue to aid maceration, and transferred to a flask, 10 cc. of distilled water being used for each gm. of tissue. When the amount of tissue was small, 20 or 50 cc. per gm. were used, as noted in Fig. 1; 1 cc. of toluene per gm. of tissue was added, and the mixture shaken and allowed to stand 44 to 48 hours at room temperature (21–25°).

At the end of this period, the organ brei was passed through filter paper; enough filtrate for the determination of enzyme activity was usually obtained within an hour. The cecal and intestinal saline washings were also allowed to stand 44 to 48 hours, though it was found, for *Mustelus canis*, that a sample of the intestinal washing tested immediately gave approximately the same value as after 48 hours.[1]

4 cc. of the tissue extract, brought to a temperature of 25 ±0.2°, were added to a mixture of 2 cc. of 0.20 M sodium glycerophosphate (Eastman Kodak Company) and 2 cc. of a 3 per cent solution of sodium diethylbarbiturate kept in a test-tube at the same temperature. The tube was inverted, and a 2 cc. aliquot was removed immediately and introduced into 4 cc. of 10 per cent trichloroacetic acid for the determination of the inorganic phosphate initially present. At the end of 1 hour a second aliquot of 2 cc. was removed and added to 4 cc. of the acid. The precipitates were filtered off and the phosphorus determined in an aliquot of the filtrate by means of the Fiske and Subbarow method (7).

The pH of the hydrolyzing solutions, with 3 per cent diethylbarbiturate as buffer (8), varied from 8.6 to 8.9, falling most often at 8.7. For the skeletal extracts and the washings, 1.5 per cent buffer was found to be sufficient; this gave a pH between 8.9 and 9.1. According to Kay (6) the range 8.8 to 9.1 is optimal for the activity of mammalian phosphatases. Experiments with intestinal extracts, washings, or mixtures of these (Table I), showed about the same pH range, or slightly higher; the number of experiments was not sufficient, however, to define this region more closely.

The concentrations of the inorganic phosphate initially present in the extracts varied from 0.0 to 15.9 mg. per 100 cc. of hydrolyzing solution. The lower values were usually found in the extracts of

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[1] Longer periods of extraction, varying up to 160 hours of the skeletal tissue of several elasmobranchs did not yield preparations of greater activity.

the skeleton and the elasmobranch stomach, the higher in the kidney and cecal extracts. From the work of Kay (6) and of Martland and Robison (9) on the retardant effect of inorganic phosphate, it would appear that the differences in the concentrations of inorganic phosphate initially present in our extracts were not sufficiently large to affect the comparison of the activities of the different extracts. For the unpurified preparations used in this study, however, a strict proportionality of enzyme activity to enzyme concentration cannot be assumed.

TABLE I

Effect of pH on Activity of Fish Phosphatase

Phosphorus as inorganic phosphate liberated in 1 hour from 100 cc. of 0.05 M sodium glycerophosphate. Buffer, sodium diethylbarbiturate. Temperature 25°. Enzyme concentration, 50 per cent of extract or washing.

Narcacion nobiliana 1 Mixture of intestinal washing and extract		Mustelus canis 8 Intestinal extract		Mustelus canis 9 Intestinal washing		Mustelus canis Intestinal washing		Raia stabuliforis 1 Intestinal extract		Tautoga onitis Intestinal extract	
pH	P	pH	P	pH	P	pH	P	pH	P	pH	P
	mg.		mg.		mg.		mg.		mg.		mg.
6.5	1.3	6.6	0.2	7.1	1.7	8.2	2.5	8.1	2.3	8.0	1.2
7.1	5.3	7.1	0.5	8.1	2.8	8.3	3.8	8.2	3.0	8.1	3.0
8.2	8.0	8.1	0.5	8.4	5.7	8.5	12.0	8.3	3.6	8.4	3.1
8.5	12.6	8.4	1.2	9.0	9.5	8.6	16.6	8.5	4.0	8.6	5.0
9.1	21.4	8.5	1.7	9.3	1.0	8.8	16.9	8.7	4.5	8.7	6.1
		8.7	1.6			9.0	18.8	8.8	4.6	8.8	7.0
		9.2	1.7			9.1	20.9	8.9	5.0	8.9	10.8
						9.3	21.5	9.2	5.6	9.2	12.4
						9.5	0.9				
						9.6	0.6				

Standard of Phosphatase Activity

The standard of phosphatase activity used was the number of mg. of phosphorus liberated, as inorganic phosphate, in 1 hour from 100 cc. of 0.05 M glycerophosphate solution by a 50 per cent concentration of tissue extract at 25° and an average pH of 8.7 or 9.0 as described above.

Results

The activities of the gastrointestinal, kidney, and skeletal extracts are shown in Fig. 1 or given below. In addition to these
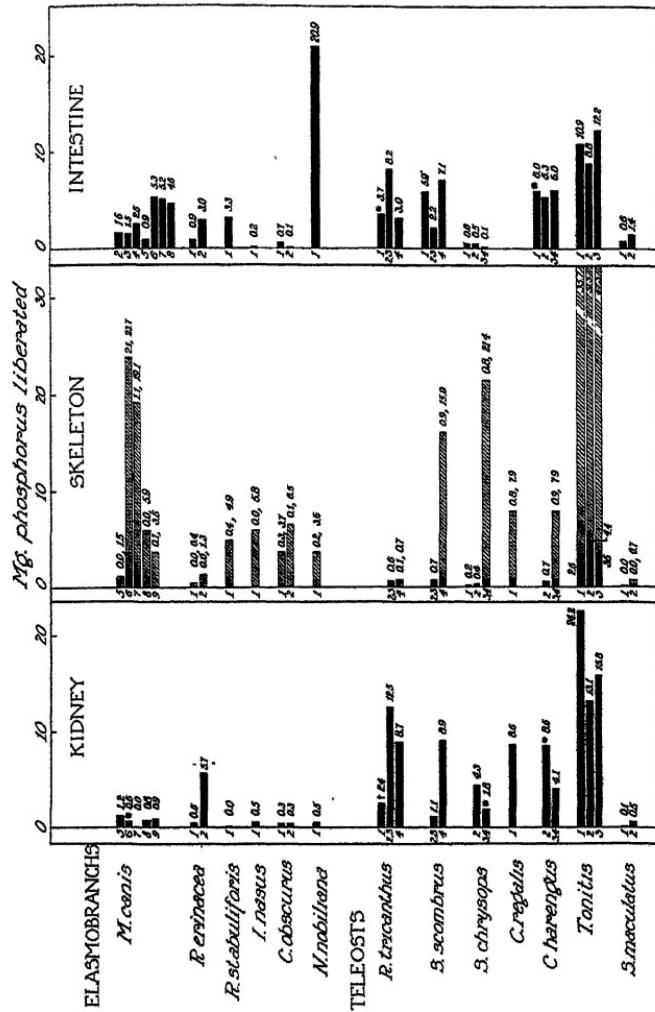
FIG. 1. The phosphatase activity of tissue extracts of elasmobranchs and teleosts. The *solid bars* represent mg. of phosphorus liberated in 1 hour from 100 cc. of 0.05 M glycerophosphate solution; the *shaded bars* represent mg. of phosphorus liberated in 25 to 27 hours. These values are given by the numbers following the bars. In the section on skeleton the first number represents the 1 hour value; the second represents the 25 to 27 hours value. The values in the section on intestine are for extracts made from the upper half or from the entire intestine; in *Stenotomus chrysops* 1 and 2, the values given are for extracts made from the ceca and intestine combined. * 20 cc. of H₂O per gm. of tissue used in extraction; † 50 cc.

tissues, the muscle, liver, and pancreas of several fish, both teleost and cartilaginous, were also tested with the same procedure. No phosphatase activity was evident within an hour.

Stomach—The stomachs of the elasmobranchs showed none or exceedingly slight activity. Thus, no phosphorus was liberated within 1 hour by either the cardiac or pyloric stomach extracts of *Mustelus canis* 2, 3, 4, 5, *Raia stabuliforis* 1, *Isurus nasus* 1, *Carcharhinus obscurus* 2. No stomach extract of any of the other elasmobranchs listed liberated more than 0.5 mg. in 1 hour.

Among the teleosts, *Clupea harengus* showed no activity. *Cynoscion regalis* showed slight activity, 0.4 mg. of phosphorus being liberated in 1 hour. Little but definite activity was found in the extracts of the other teleosts; the number of mg. of phosphorus liberated in 1 hour is as follows: *Rhombus tricanthus* 0.8, 1.2; *Stenotomus chrysops* 2.0, 2.8, 0.9; *Spheroides maculatus* 0.3, 3.0; *Scomber scombrus* 0.1, 1.1, 1.6. *Stenotomus chrysops* showed most activity. The stomach of this fish, not pronouncedly saccular, was identified by the change in the appearance of the mucosa and by its position cephalad to the bile duct opening. It showed greater phosphatase activity than did the intestine.

Pyloric Ceca—With the exception of *Stenotomus chrysops*, teleosts possessing pyloric ceca yielded very active extracts. The number of mg. of phosphorus liberated in 1 hour is as follows: *Rhombus tricanthus* 7.4, 6.1; *Scomber scombrus* 3.4, 5.4, 8.3; *Cynoscion regalis* 6.9; *Clupea harengus* 9.0, 5.7, 6.6; *Stenotomus chrysops* 0.8, 0.6, 0.5.[2]

Intestine—In general, the elasmobranchs showed less activity than did the teleosts. Among the elasmobranchs, the order of increasing activity was (1) *Isurus nasus, Carcharhinus obscurus;* (2) *Mustelus canis, Raia stabuliforis;* (3) the very small dogfish, *Mustelus canis* 6 and 7; (4) *Narcacion nobiliana*. The activity of *Narcacion nobiliana* was the greatest recorded in the entire series of fish. Among the teleosts, *Tautoga onitis* showed unusually great activity; *Stenotomus chrysops* and *Spheroides maculatus*, very little. The others gave extracts of considerable activity. In many instances, the intestinal tract was divided in half, into an upper and lower region, but no regular difference in activity was

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[2] The last two values for *Stenotomus chrysops*, 0.6 and 0.5 mg., are for extracts made from ceca and upper half of intestine, combined.

observable between these regions. In *Tautoga onitis* the bile duct opened apparently at the most cephalad end of the gastro-intestinal tract and no region was grossly identifiable as the stomach; the tract was divided into three equal parts, all of which proved to be very active.

Kidney—The distinction between the elasmobranchs and the teleosts was striking. Eleven of the twelve elasmobranchs showed very little activity; these included all the species tried. One individual, *Raia erinacea* 2, yielded an active extract. On the other hand, all the teleosts, with the exception of the species *Spheroides maculatus*, showed considerable activity.

Skeleton—In general, the elasmobranchs showed less activity than the teleosts. With the exception of those of the dogfish pups, *Mustelus canis* 6 and 7, the elasmobranch extracts liberated 0.0 to 0.4 mg. of P in 1 hour. In contrast to this, the teleost extracts, with the exception of *Spheroides maculatus*, liberated 0.2 to 4.4 mg. of P, usually about 0.7 mg. of P. The activities of the extracts of the dogfish pups were unusually high, 2.1 and 1.1 mg. of P being liberated in 1 hour. In several cases, samples of the hydrolysis mixture were also taken at the end of 25 to 27 hours. The values obtained are shown as the shaded bars in Fig. 1. They emphasize the differences obtained with the 1 hour samples.

Cecal and Intestinal Washings—10 cc. of saline per gm. of tissue were ordinarily used in washing. The activity of these washings was, in general, weak and showed little relation to the activity of the extract of the corresponding tissue. In these cases, about 0.5 to 2.0 mg. of P were liberated in 1 hour. In three species, however, the findings were different. In *Tautoga onitis*, the intestinal extracts were very active in all three individuals tested; in contrast to this, eight of the nine washings showed no activity at all; the other washing was very weak. In *Narcacion nobiliana* very active washings (9.5 mg. for the upper intestinal washing and 18.2 mg. for the lower) corresponded to the similarly active extracts. In *Mustelus canis*, the washings were usually quite active (5 to 10 mg. of P liberated in 1 hour) even though some of the corresponding extracts were weak.

On the whole, the tissues of the teleosts showed greater activity than those of the elasmobranchs. The difference was most pronounced in the kidney and least in the intestine. Among the

teleosts there was also a rough correspondence in the individual between the activities of the three tissues; a high intestinal phosphatase, for instance, was usually accompanied by a high bone and a high kidney phosphatase.

Robison and his coworkers (1) consider that the phosphatase in bone and ossifying cartilage effects the hydrolysis of certain phosphoric esters which are present in the blood; the concentration of inorganic phosphate in the tissue fluid is thereby increased and a calcium phosphate compound is deposited. Kay (2) recently reviewed the evidence that had been presented for this hypothesis.

Certain of this evidence aims particularly to establish a correlation between the presence of phosphatase in bone and histological signs of ossification. Thus Robison (10) reported phosphatase to be absent from the non-ossifying tracheal cartilage and Martland and Robison (11) found that the first appearance of the enzyme in the cartilages and bones from human embryos and young infants coincided with the appearance of the ossification center. Fell and Robison (12) have shown that in embryonic avian femora and the embryonic mandibular skeleton cultivated *in vitro* the synthesis parallels the histological differentiation of ossification.

The microscopic structure of the endoskeletal tissue of fish may be of three kinds (13): cartilaginous, dentine-like, bony. The first is characteristic of elasmobranchs; calcium salts may be deposited but there is no evidence of bone cells or of bone substance formation. The dentine-like structure, present in certain Teleostomi and Dipnoi, is characterized, most observers agree, by the absence of bone cells from the matrix. The cells are arranged in rows at the periphery (14). Fine canals run through the ground substance and contain prolongations of the processes of the peripheral cells. According to Kölliker (15), who first made extended studies on the distribution of this type of structure, it occurs in the following orders: Acanthopteri (*Thynnus* excepted), all Anacanthini, Pharyngognathi, some Physostomi, the Plectognathi, and the Lophobranchii. The third type of tissue in which the bone cells are present in the matrix occurs among the higher tribes of Physostomi, all the Ganoidei, Sirenoidei, and the genus *Thynnus* of the Acan-

thopteri.   Of the teleosts used in the present work, only *Clupea harengus* falls into this third group.

In this study phosphatase was found to be present in the endoskeletal tissue of the cartilaginous as well as the bony fish.   These results, therefore, do not support the assumption of a necessary association between the presence of the enzyme and the occurrence of ossification.   In general, a higher skeletal phosphatase content was found in the teleosts than in the elasmobranchs, but to regard this higher content as determinative of ossification in the teleosts would leave unexplained the still higher values found in the dogfish pups and the very low values found in *Spheroides maculatus*.[3]

With regard to the rôle of kidney phosphatase, Eicholtz, Robison, and Brull (17) suggested that urinary phosphate is largely, if not entirely, derived from the organic phosphates of the plasma by the action of that enzyme.   Later Kay (18) showed that kidney phosphatase preparations liberated inorganic phosphate from rabbit and human plasma.   He calculated the amount of inorganic phosphate which could be formed in this way by the human kidney and stated the limiting factor in such production to be not the amount of enzyme but the amount of hydrolyzable substrate in the plasma.

In view of Kay's suggestion, it would be interesting to correlate the kidney phosphatase activity with the excretion of urinary phosphate in individuals among the elasmobranchs.   The values reported in this paper for *Mustelus canis* are not sufficiently high, according to Kay's method of calculation, to account for the average urinary phosphate excretion as given by Denis (19).

For a pooled lot of urine of *Mustelus canis*, Denis' analysis gave 4.52 gm. of P_2O_5 per liter.   The average daily volume excretion was 12 cc. for a kilo dogfish and the phosphorus excretion, therefore, 24 mg. of P per day, or 1 mg. per hour.   The values given in

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[3] *Spheroides maculatus* belongs to the family Tetraodontidæ, order Plectognathi.   The softness of the bones of members of this order has often been noted.   According to Stephan (16), the structure corresponding to this physical state is a network of fine osseous trabeculæ and a groundwork of a hyaline but non-cartilaginous substance; the whole is alveolar in appearance.   The compactness of the bone increases as one passes from Molidæ to Tetraodontidæ, the last resembling other teleosts most closely in this respect.

Fig. 1 are on the basis of 50 cc. of kidney extract or 5 gm. of kidney. According to our experience, this would be the weight of the kidney in a dogfish weighing 1 kilo. At pH 7.3 to 7.4, Kay calculates the phosphatase activity to be one-third to one-half of that at the optimal pH. Accordingly, the phosphatase in the kidney of a dogfish (*Mustelus canis*) weighing 1 kilo is capable of liberating about 0.0 to 0.6 mg. of P per hour.

SUMMARY

1. The distribution of phosphatase in the various portions of the gastrointestinal tract, in the kidney, and endoskeleton has been studied in teleosts and elasmobranchs.

2. The phosphatase activity of these tissues in the teleosts studied is, on the whole, greater than that of the corresponding tissues in the elasmobranchs.

3. Phosphatase occurs in the cartilaginous skeleton of the elasmobranch as well as in the dentine-like or bony skeleton of the teleost.

BIBLIOGRAPHY

1. Robison, R., and his collaborators in many articles appearing in *Biochem. J.*, 1923–1931.
2. Kay, H. D., *J. Biol. Chem.*, **89**, 249 (1930).
3. Heymann, W., *Z. Kinderheilk.*, **49**, 748, 761 (1930).
4. Page, I. H., and Reside, D. M., *Biochem. Z.*, **223**, 171 (1930).
5. Page, I. H., *Biochem. Z.*, **223**, 222 (1930).
6. Kay, H. D., *Biochem. J.*, **22**, 855 (1928).
7. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
8. Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).
9. Martland, M., and Robison, R., *Biochem. J.*, **21**, 665 (1927).
10. Robison, R., *Biochem. J.*, **17**, 286 (1923).
11. Martland, M., and Robison, R., *Biochem. J.*, **18**, 1354 (1924).
12. Fell, H. B., and Robison, R., *Biochem. J.*, **23**, 767 (1929); **24**, 1905 (1930).
13. Dean, B., Bibliography of fishes, New York, **3**, 588 (1923).
14. Stéphan, M. P., *Compt. rend. Soc. biol.*, **5**, 551 (1898).
15. Kölliker, A., *Proc. Roy. Soc. London*, **9**, 656 (1859).
16. Stephan, P., *Bull. sc. France et Belg.*, **33**, 280 (1900).
17. Eicholtz, F., Robison, R., and Brull, L., *Proc. Roy. Soc. London, Series B*, **99**, 91 (1925).
18. Kay, H. D., *Biochem. J.*, **20**, 791 (1926).
19. Denis, W., *J. Biol. Chem.*, **13**, 225 (1912–13).

A METHOD FOR THE DETERMINATION OF HEXOSE-MONOPHOSPHATE IN MUSCLE

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The observation, that more muscle glycogen disappeared after epinephrine injection than could be accounted for as lactic acid (1), suggested that a substance intermediary between glycogen and lactic acid accumulated in muscle under these conditions. In the course of the investigation of this problem the need was felt for a method permitting the separation of hexosemonophosphate from other phosphorus compounds present in muscle and its simultaneous determination as reducing substance and as P. A method embodying these features is described below.

In 1927 Embden and Zimmermann (2) isolated a hexosemono-phosphate—frequently referred to as Embden ester or as lactacidogen—from fresh rabbit muscle. "Lactacidogen" was originally supposed to be a hexosediphosphate which Embden and Zimmermann (3) had isolated previously from muscle press-juice. When it was recognized that hexosediphosphate does not occur in intact muscle, the term lactacidogen was applied by Embden to the hexosemonophosphate. Unless stated to the contrary, hexosephosphate as used throughout this and the following paper (4), refers to the Embden monoester. A description of the chemical properties of this ester (and of other biological esters) such as composition, optical rotation, reducing power for various sugar methods, rate of splitting off of H_3PO_4 during acid hydrolysis, dissociation constants, solubility of various salts, and other properties, has recently been given by Lohmann (5). Much information about hexosephosphates may also be found in Meyerhof's (6) book. In 1921 (7) and again in 1928 (8) Embden and his collaborators described a method for the determination of "lactacidogen," which will be discussed later.

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Distribution of Acid-Soluble P Compounds in Muscle—A trichloroacetic acid extract of the gastrocnemius muscle of the rat contains on an average 190 mg. of P per 100 gm. of muscle. About 110 mg. or 58 per cent are inorganic plus phosphocreatine P, 64 mg. or 34 per cent are adenosinetriphosphate P, and 9 mg. or 5 per cent are hexosemonophosphate P, leaving about 7 mg. or 3 per cent of undetermined P. Adenosinemonophosphate (also referred to as adenine nucleotide or adenylic acid) and its deamination product, inosinic acid, seem to be present in traces only in a fresh trichloroacetic acid extract of resting muscle, as may be judged from the fact that the pentose content is almost theoretical for adenosinetriphosphate. For example, in an extract hydrolyzed for 7 minutes in N HCl at 100° there were formed 46 mg. of inorganic P, corresponding to 69 mg. of adenosinetriphosphate P per 100 gm. of muscle (see p. 575). From this a pentose content of the extract of 111 mg. per 100 gm. of muscle may be calculated, while the pentose content as actually determined (by means of the orcinol-HCl-FeCl₃ color reaction of Bial with adenine nucleotide as standard) was 114 mg. In two other experiments the calculated pentose content was 104 and 119 mg. per 100 gm. of muscle, while the pentose found was 106 and 126 mg. respectively. It is apparent that most of the pentose present is contained in the adenosinetriphosphate and that there is little room left for the presence of adenylic or inosinic acid.

Changes in P Compounds during Standing of Extract—When the trichloroacetic acid extract (prepared with 5 per cent acid) is allowed to remain at room temperature (17–24°) for 16 to 20 hours, phosphocreatine is split almost completely into its components, while no detectable amount of hexosephosphate is lost and only 2 to 4 mg. of adenylic or inosinic acid P are formed from adenosinetriphosphate. Phosphocreatine, adenylic acid, and inosinic acid form soluble barium salts and for this reason the presence of these compounds in more than traces is a disturbing factor in the execution of the method.

The amount of adenylic or inosinic acid P (in mg. per 100 gm. of muscle) formed in a trichloroacetic acid extract kept for 17 hours at different temperatures, was as follows: 7°, 1.5 mg.; 24°, 3.5 mg.; 29°, 5.5 mg.; 38°, 7 mg. These values were calculated from the difference in the amount of P liberated during 7 minutes hydrol-

ysis at 100° in N HCl in the fresh extract and after standing.   In the sample kept at 7° 6.3 mg. of phosphocreatine P were present, while the sample kept at 24° contained 1 mg.   Whereas the hexose-phosphate precipitate prepared from the trichloroacetic acid extract kept at 7° gave no pentose reaction with phloroglucinol-HCl, a positive test, indicating admixture of adenylic or inosinic acid, was obtained with the hexosephosphate precipitate prepared from the extract which had stood at 38°.   The presence of small amounts of phosphocreatine in the extract is less objectionable than that of adenylic acid, because it is easy to apply a correction for an admixture of the former compound.   The best temperature at which to keep the extract is 15°, since very little adenylic acid is formed, while most of the phosphocreatine is split.   If this is not possible, the extract may be kept for a few hours at a higher temperature and then placed in the refrigerator overnight.

Separation of Hexosephosphate from Other P Compounds—By adjusting the extract which has stood overnight to pH 8.2 by means of barium hydroxide, inorganic phosphate, adenosinetri-phosphate, and the fraction containing undetermined P are almost completely precipitated, while 60 to 70 per cent of the hexosephos-phate originally present remains in solution.   In order to recover that part of the hexosephosphate which is carried down, presumably as a double salt, with the precipitate, it is necessary to decompose the precipitate with sulfuric acid; washing the precipitate is ineffective.   After centrifuging off the barium sulfate, the super-natant fluid is again brought to pH 8.2 by means of barium hydrox-ide.   In this manner a good separation of hexosephosphate from other phosphorus compounds is effected and the recovery is about 93 per cent.   If the barium precipitate is decomposed a second and third time, some additional hexosephosphate is recovered, but the gain is too small to make it worth while.   Three examples of the amount of hexosephosphate (in mg. of hexose per 100 gm. of muscle) lost if the barium precipitate is not decomposed, are given below.[1]

[1] Eggleton and Eggleton (9) in an attempt to separate the different P compounds in muscle, made the trichloroacetic acid extract faintly alkaline to phenolphthalein but did not decompose the barium precipitate.

Decomposed	64.0	89.9	110.5
Not decomposed	44.8	62.2	70.1
	−19.2	−27.7	−40.4
Loss in per cent	29.6	30.8	36.5

The reason for choosing pH 8.2 is made clear by the experiments in Table I. It will be noted that at a pH more acid than 8.2 a considerable amount of inorganic phosphate remains in solution, while at the alkaline side of pH 8.2 there is occasionally a slight

TABLE I

Yield of Hexosephosphate and Admixture of Inorganic P at Different pH

A trichloroacetic acid extract of rat muscle was adjusted to different pH with barium hydroxide and then carried through the method as described in the text.

Hexosephosphate is expressed as mg. of hexose per 100 gm. of muscle and inorganic P is given in mg. per 100 gm. of muscle.

pH	7.4	7.6	7.8	8.0	8.2	8.5	8.7	8.9
Hexosephosphate:								
Experiment 1	48.1	47.2	46.0	44.4	43.5			38.8
" 2				54.1	54 0	54.1	53.4	51.0
" 3	77.5	75.9	75.0	76.7	73.8			66.8
" 4	131.5	131 5	122.0	124.2	124.1			
" 5		146.0	151.3	148.2	148.0			
" 6				186.1	189.0	182.2	187.5	178.0
Inorganic P:								
Experiment 1	15.1	8.9	7.9	5.7	4.2			2.5
" 3	23.9	19.1	13.3	7.6	5.4			2.4
" 4	12.5	8.2	6.8	4.9	3.8			
" 5		9.9	5.9	5.4	4.4			2.8

loss of hexosephosphate. This is true whether the hexosephosphate content of muscle is high or low. Some inorganic P (from traces of phosphocreatine?) is present, even if the pH is adjusted to pH 8.9 and this makes it necessary to determine in every experiment the amount of inorganic P included in the hexosephosphate precipitate in order to apply a correction.

It would seem advisable to make preliminary experiments of the type shown in Table I in order to find the optimal pH values, whenever the method is applied to muscle of other species or to heart

muscle, because there is the possibility that these tissues may not behave in exactly the same way as does rat muscle.

Separation of Hexosephosphate from Other Reducing Substances— As far as is known, the chief interfering substances in an acid extract of muscle are creatine, glutathione, and simple sugars. Creatine has a reducing intensity for alkaline ferricyanide only one-tenth of that of glucose, but nearly 0.5 per cent of it is present in muscle. The tripeptide reduces ferricyanide one-half as much as glucose and is present to the extent of about 30 mg. per 100 gm. of muscle. Finally there are about 20 to 30 mg. of fermentable sugar present per 100 gm. of muscle. When hexosephosphate, after its separation in the manner described above, is precipitated as barium salt in 80 per cent alcohol, creatine and fermentable sugars remain in solution. That creatine and glucose do not interfere under these conditions was shown by adding known amounts of them to a trichloroacetic acid extract of muscle; the reducing value obtained for hexosephosphate was the same as in the trichloroacetic acid extract to which no creatine and glucose had been added. When this experiment was repeated with glutathione, it was found that part of the tripeptide added was included in the hexosephosphate precipitate. It was calculated that the error introduced by glutathione can amount at the most to 10 mg. per 100 gm. of muscle in terms of hexose. A separation of glutathione and other nitrogenous substances from hexosephosphate by means of mercuric or lead acetate could not be accomplished without a considerable loss of the ester.

Reducing Power of Embden Ester—According to Embden and Jost (8) the ester has a reducing power 67 per cent of that of glucose, when the ferricyanide reagent of Hagedorn and Jensen is used. An identical value was reported by Lohmann (5). Several samples of the barium salt of the Embden ester prepared in this laboratory from rabbit and rat muscle gave a ratio of P to hexose corresponding to the above reducing power. One example for this is given below.

A solution contained 0.40 mg. of $C_6H_{11}O_5 \cdot PO_4Ba$ per cc.

mg.

Reducing power per cc. (in terms of glucose) 0.117

Hexose content   "   "   (0.117 × 1.5) 0.1755

P content calculated per 3 cc. $\left(\dfrac{0.1755 \times 3}{5.8} \right)$ 0.0907

The reducing power of the purified Embden ester for the Benedict copper reagent (10) is very similar to that obtained for the ferricyanide reagent. Crude Embden ester (*i.e.*, ester as separated in the execution of this method) contains admixtures of substances of an unknown nature which inhibit the reduction of the Benedict reagent. These substances can be removed by means of mercuric and lead acetates. With the Shaffer-Hartmann reagent as modified by Somogyi (11) the reducing power of purified as well as of "crude" ester was 59 per cent of that of glucose.

Within certain limits the reducing power of the Embden ester for ferricyanide is proportional to the amount present, as is illustrated by the following examples. The values read off in the original table of Hagedorn and Jensen were multiplied by 1.5.

A solution contained 0.051 mg. of purified Embden ester (in terms of hexose) per cc.

		mg.
Per 2 cc. found		0.105
" 3 " "		0.157
" 4 " "		0.206
" 5 " "		0.255

A solution contained 0.0795 mg. of "crude" Embden ester (in terms of hexose) per cc.

		mg.
Per 2 cc. found		0.159
" 3 " "		0.227
" 4 " "		0.290
" 5 " "		0.354

In the case of the purified ester the proportionality in reducing power was satisfactory for amounts ranging from 0.05 to 0.25 mg. The "crude" ester showed a falling off in reducing power when more than 0.2 mg. was present. Attention must be given to this fact in the execution of the method; *i.e.*, the amount to be determined should be below 0.2 mg. in terms of hexose when the table of Hagedorn and Jensen is used, otherwise it is necessary to construct a separate table for hexosephosphate.

Removal of Muscle from Living Animal—The procedure frequently resorted to of killing an animal by stunning, bleeding, or administration of ether, chloroform, or coal gas, prior to the removal of muscle for analysis, has been found objectionable in the case of glycogen and lactic acid determinations in muscle. It

is equally objectionable in the case of hexosephosphate determinations in muscle. In order to obtain resting values for hexosephosphate it is necessary to remove the muscles from the living anesthetized animal and to chill the muscle at once. Freezing the muscle *in situ* was not found to be of special advantage.

The procedure adopted in the case of the rat is as follows: 10 cc. of 5 per cent trichloroacetic acid are pipetted accurately into a 15 cc. centrifuge tube or test-tube of similar dimensions. The tube is stoppered with a cork and placed in crushed ice. When cold, the tube is wiped with a towel and weighed on a semianalytical balance to the nearest 5 mg. If the moisture which accumulates on the cold tube is wiped off again with a towel before the final adjustment of weight is made, the error introduced by this factor is small. Shortly before the muscle is taken for analysis, the animal is given an intraperitoneal injection of amytal (10 mg. per 100 gm.). When anesthesia is complete, *i.e.* in about 5 to 10 minutes, the gastrocnemius is exposed and separated from adjacent muscles by blunt dissection. After the Achilles tendon is cut the muscle is lifted up and severed by one thrust of the scissors at its insertion in the popliteal region. The muscle is submerged at once in the ice-cold acid and its weight is ascertained by weighing the tube back after it has stood for a few minutes on ice. Acid and muscle are now transferred to a mortar and the muscle is crushed at once with the pestle. 1 to 2 gm. of acid-washed quartz sand are added and the whole is ground to a very fine state of division. This is very important because otherwise the extraction is incomplete.

1:10 Muscle Filtrate—It is convenient for subsequent calculations to make an exactly 1:10 dilution. The water content of muscle is assumed to be 80 per cent. If for instance the muscle weight happens to be 2.5 gm., 2 cc. of water are contained in the muscle; 10 cc. of acid have already been added, so that an additional 13 cc. of acid are required to give 25 cc. or a 1:10 dilution. When all the acid has been added, the contents of the mortar are thoroughly mixed and after standing for 10 minutes the whole mass is filtered. The filtrate must be water-clear under all conditions and must not get turbid on standing; it is kept overnight, preferably at 15°, or it may be kept for 6 hours at 20–24° and then overnight in the ice box.

pH Adjustment with Barium Hydroxide—On the following day 15 cc. of the filtrate—or any other suitable volume—are pipetted into a 25 cc. centrifuge tube and 1 drop of indicator solution[2] (cresol red) is added. Oven-dried and finely powdered barium hydroxide is added and worked with a glass rod in the tip of the tube. When most of it is in solution more is added until a precipitate begins to form. Small portions of barium are now cautiously added and rubbed up with the glass rod until the pH of the solution is close to but still on the acid side of pH 8.2.[3] An excess of undissolved barium hydroxide should be avoided. With a little practice these operations can be carried out expeditiously. Should the end-point have been exceeded, a drop or two of trichloroacetic acid is added until the solution is of the desired pH.

The barium precipitate is centrifuged off and the supernatant fluid is poured into a 20 cc. volumetric flask. To the barium precipitate are added 4 cc. of water and about 5 to 7 drops of N sulfuric acid, so that the reaction is distinctly acid to Congo paper. The barium sulfate is centrifuged off and the clear supernatant fluid is tested for barium with a small drop of 0.1 N sulfuric acid. If no precipitate forms the supernatant fluid is poured into a 15 cc. centrifuge tube and 1 drop of indicator solution (of one-half the strength of that used above) is added. The solution is brought to pH 8.2 by addition of barium hydroxide—only a small amount is needed—and the precipitate is centrifuged off. The supernatant fluid is added to the one already present in the 20 cc. volumetric flask, and now the final pH adjustment is carried out by adding drop by drop a very dilute barium hydroxide solution and matching the color against a standard buffer solution of pH 8.2 containing the same concentration of indicator. When the desired color has been reached, the flask is made up to the mark and the contents are mixed. A slight precipitate is centrifuged off

[2] 100 mg. of cresol red are rubbed up in a mortar with 5.3 cc. of 0.05 N sodium hydroxide and made up to 100 cc. An aliquot part is diluted four times to give a 0.025 per cent solution.

[3] A standard buffer solution of pH 8.2 is prepared by mixing 96.8 cc. of M/15 Na_2HPO_4 with 3.2 cc. of M/15 KH_2PO_4. This mixture keeps only for a few days in the ice box. The M/15 stock solutions are prepared from "Sörensen" salts and may be stored for some time in Pyrex flasks in the refrigerator. A good buffer solution for this pH region is the sodium acetate-diethylbarbiturate mixture recently described by Michaelis (12).

in a 25 cc. tube. This tube is then placed next to a similar tube containing buffer solution of pH 8.2 and both are held against a white background for a final comparison of the colors.

Most of the experiments reported in this and in the following paper were made by adjusting to a pH of 8.2. It was found later that phenolphthalein may be used as indicator, which obviates the use of a standard buffer solution. Since the titration exponent (Bjerrum) of a one color indicator such as phenolphthalein depends very considerably upon its concentration, the following conditions should be adhered to. To 15 cc. of the trichloroacetic acid filtrate are added 4 drops of a 0.1 per cent solution of phenolphthalein in 50 per cent alcohol. The filtrate is brought to a very faint pink by means of barium hydroxide, pH about 8.6. After decomposing the barium precipitate 1 drop of the above indicator solution is added. The final pH adjustment is to a very faint pink, otherwise the method is the same as described above.

Precipitation with Alcohol—18 cc. of the above solution are precipitated with alcohol. It is convenient to pipette four 4 cc. samples into 25 cc. centrifuge tubes and one 2 cc. sample into a graduated 15 cc. centrifuge tube. 96 per cent alcohol (containing 3 cc. of ammonia, sp. gr. 0.9, per liter) is added, 20 cc. to the 25 cc. centrifuge tubes and 10 cc. to the 15 cc. tube. The alcohol concentration is thus close to 80 per cent. The contents are thoroughly mixed and the tubes are placed for 2 hours in the refrigerator.[4] A flocculent precipitate forms which is centrifuged off; at least 2000 revolutions per minute and a centrifugation time of 10 to 15 minutes are required to effect a complete separation. It is desirable to hold the tubes against a dark background and to turn them slightly in order to make sure that there are no floating particles left. The alcohol is poured off,[5] the tubes are drained on filter paper and placed in a vacuum desiccator over sulfuric acid or some other convenient drying agent, and the desiccator is evacuated. The precipitate should not be dried too much, be-

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[4] The question whether the tubes can be left in the ice box overnight has not been sufficiently investigated to recommend such a procedure.

[5] The total P content of the alcohol which is poured off was found to be about 1 mg. per 100 gm. of muscle. This P might belong to some other compound. Even if it were hexosephosphate P the loss, due to solubility of the latter in 80 per cent alcohol, would be very slight.

cause it is then more difficult to bring it into solution. The tubes are merely left in the desiccator long enough to drive off the last traces of alcohol; $\frac{1}{2}$ hour is generally sufficient if a high vacuum is maintained.

Determination of Reducing Power and P Content—The precipitate in the tubes is dissolved in 3 to 4 cc. of 0.1 N HCl. The acid is added to one tube, the precipitate is stirred up with a glass rod, and the contents are transferred to the next tube and so on until all has been collected in the graduated 15 cc. centrifuge tube. The tubes are then washed twice in the same manner with 2 cc. of water. When all the washings have been collected in the 15 cc. tube, enough sulfuric acid is added to precipitate the barium completely, about 3 to 4 drops of N acid sufficing. A large excess of the acid is to be avoided because otherwise an appreciable "salt error" is introduced in the sugar determination. When after the addition of a small drop of dilute sulfuric acid no further precipitation occurs, the tube is made up to the 8 or 10 cc. mark, depending on the amount of hexosephosphate to be expected, and after centrifuging off the precipitate the contents are poured into another graduated 15 cc. centrifuge tube. In order to neutralize the solution without appreciably changing the volume, the tip of a fine glass rod is dipped into a saturated solution of NaOH. The small amount of fluid adhering to the glass rod is generally sufficient to make the solution neutral to litmus paper.

Two samples of 1 cc. each are used for the sugar determination and 4 cc. are ashed for the total P determination. The remainder is used for a determination of inorganic P which is always present in small amounts (see Table I) and for which a correction must be applied. For the P determination the Fiske-Subbarow method (13) is used. The standard, containing 0.08 mg. of P, is made up to 15 cc., which allows the standard to be read against itself for a setting of our colorimeter at 20. The ashed sample is made up to the same volume; graduated 15 cc. centrifuge tubes were found very convenient and sufficiently accurate for that purpose. The ashing is carried out in the usual manner in a thick walled Pyrex tube; *i.e.*, the full amount of sulfuric acid which is needed later for the development of the color—in our case 1.5 cc. of 5 N acid—is added and the solution is boiled down on an asbestos mat until the material is charred and white fumes make their appearance.

The tube is then removed from the flame and after cooling a bit, 1 or 2 drops of phosphate-free hydrogen peroxide are added. Heating is resumed and if the solution does not become water-clear, the addition of peroxide is repeated. Two to three additions of peroxide are generally sufficient. As much as possible of the peroxide is driven off in the last heating and after cooling and addition of water the solution is boiled for $\frac{1}{2}$ minute in order to hydrolyze traces of metaphosphoric acid. The contents are then transferred to a graduated 15 cc. centrifuge tube and the sample is ready for the colorimetric P determination. In the meantime the volume of fluid left for inorganic P determination has been read off and 1.5 cc. of 5 N sulfuric acid have been added. This destroys traces of phosphocreatine, if present. Since the inorganic phosphate content is too low to be read directly, 0.08 mg. of P is added before the color is developed.

The reducing power is determined by means of the ferricyanide reagent of Hagedorn and Jensen. Since this reagent is not perfectly stable, a determination on a standard glucose solution (prepared from Bureau of Standards glucose) is carried out every 2nd and 3rd day and a correction for any deviation from the standard (generally not more than 4 per cent) is applied. A blank determination on the reagents is carried out every day. The blank has been found to vary between 0.002 and 0.007 mg. in terms of glucose and is deducted in every case.

The method itself yields a small blank. This may be shown by treating 5 per cent trichloroacetic acid containing inorganic phosphate in exactly the same way as a muscle extract. The alcohol precipitate (mostly barium hydroxide) is dissolved in the same quantity of HCl as when carrying out the method and after removal of barium with sulfuric acid and neutralization, 0.1 mg. of glucose per cc. is added. The values obtained varied between 0.101 and 0.104 mg. of glucose per cc. No correction was applied for this blank.

In calculating the results, the reducing value for ferricyanide corrected for reagent blank and deviation from glucose standard and multiplied by 1.5 gives the hexose content of the sample and the latter value divided by 5.8 gives the theoretical P content for Embden hexosemonophosphate. The calculated P value is then compared with the P value obtained after ashing and

after deducting the correction for inorganic P.   Numerous examples illustrating the extent of agreement between P calculated and P found are shown in Table III of this and in Tables I to VI of the following paper (4).   If the P value, as determined, is appreciably higher than the calculated P value, this is generally due to the presence of adenylic or inosinic acid, which form soluble barium salts but have practically no reducing properties.

Recovery of Added Embden Ester—Known amounts of Embden ester were added to a trichloroacetic acid extract of muscle or to 5 per cent trichloroacetic acid containing about the same concentration of phosphates as the muscle extract and the method was

TABLE II

Recovery of Embden Ester Added to Trichloroacetic Acid Extract of Muscle

The values are expressed as mg. of hexose.

Extract No.	Present in 8 cc.	Amount added	Recovered of amounts added	
	mg.	*mg.*	*mg.*	*per cent*
1	0.35	0.37	0.38	102.8
	0.35	0.73	0.66	90.4
2	0.59	0.60	0.52	87.5
	0.59	1.47	1.34	90.5
		0.36*	0.34	94.5
		0.63*	0.58	92.1
Average...				92.9

* Added to 8 cc. of 5 per cent trichloroacetic acid containing 0.02 per cent P (as inorganic phosphate).

carried out as described above.   An average of 92.9 per cent was recovered (Table II).   In order to recover more than 93 per cent it is necessary to decompose the barium precipitate a second and third time, but as mentioned before, this seems hardly worth while.

Method of Embden and Jost—In this method (8) the acid extract of muscle (made with a solution containing 5 per cent trichloroacetic and 1 per cent hydrochloric acid) is made alkaline to phenolphthalein with magnesium oxide and after centrifuging off the excess magnesium oxide the supernatant fluid is twice precipitated with alcohol.   The reducing value obtained by means of the Hagedorn-Jensen method on the dissolved alcoholic precipitate is

taken as a measure of the hexosephosphate content of the sample. It is to be noted that the alcoholic precipitate contains from 50 to 70 mg. of P per 100 gm. of muscle, while the hexosephosphate P of resting muscle is only 8 to 10 mg. A separation of hexosephosphate from other phosphorus compounds in muscle has not been effected and it is therefore impossible to evaluate the content of the former from total P determinations. This is a disadvantage of the method because without simultaneous P determinations one cannot be certain that changes in reducing power are due to changes in hexosephosphate content.

Furthermore, the presence of a large amount of magnesium phosphate and carbonate in the solution to be analyzed for its reducing power by means of a sensitive micro method is objectionable, because a considerable error is introduced in this manner. This is shown in the following experiments. The trichloroacetic-hydrochloric acid solution containing the same concentration of phosphate as a muscle extract was treated in exactly the same way as in the method of Embden and Jost. To the dissolved precipitate a known amount of glucose was added; instead of finding the theoretical value of 0.1 mg. per cc., the results obtained in four different experiments were 0.120, 0.117, 0.116, and 0.118 mg. per cc. Two samples of c.p. magnesium oxide were used, so that this result cannot be attributed to impurities in that chemical. When the above experiment was repeated with 5 per cent trichloroacetic acid containing no hydrochloric acid, less magnesium was contained in the precipitate and in consequence of this the error introduced in the sugar determination was smaller, as shown by values of 0.110 and 0.112 mg. per cc.

On several occasions recovery experiments of the type shown in Table II were made with the method of Embden and Jost. Within a certain range the recovery of added hexosephosphate was seemingly satisfactory because the loss of hexosephosphate was approximately balanced by the increase in reducing power brought about by the magnesium salts. After applying a correction for the error introduced by the magnesium salts, the recovery was only 70 to 75 per cent.

Influence of Autolysis on Phosphorus Compounds in Muscle—In Embden's (7) original method for the determination of "lactacidogen," minced muscle was suspended in 1 per cent sodium bicarbo-

nate and incubated for 2 hours at 37°. Inorganic P was determined before and after incubation and the difference between these two values was assumed to be phosphorus split off from hexosediphosphate under the influence of an enzyme. Embden (2) found later that the carbohydrate ester occurring in muscle is a hexosemono- and not a hexosediphosphate. Shortly afterwards Lohmann (14) showed by means of hydrolysis curves in N HCl before and after incubation that most of the P split off during autolysis of frog or rabbit muscle comes from a compound which, unlike hexosemonophosphate, is easily hydrolyzed in N HCl and which he identified as pyrophosphate.[6] This was confirmed by Embden (8) and led to the abandonment of the old lactacidogen method.

According to Lohmann's (19) analysis a small portion of the P liberated during autolysis of muscle comes from a compound which is difficult to hydrolyze in N HCl and which he believes to be mainly hexosephosphate because he found that Embden ester added to minced muscle was completely split during 2 hours of incubation. In fact, Lohmann assumed that the difference between total autolyzable P and pyrophosphate P could be used as a measure of the hexosephosphate content of muscle. In the experiments in Table III to be described below a somewhat different conclusion was arrived at.

Several muscles were removed from a rat immediately after death and cut into small pieces. After thorough mixing one portion was weighed and was placed at once in 5 per cent trichloroacetic acid. The other weighed portion was rubbed up in a mortar in 3 times the volume of 1 per cent sodium bicarbonate and kept for 2 hours at 37°. A 1:10 trichloroacetic acid filtrate was then prepared in the usual manner. Hexosephosphate, inorganic plus

[6] Lohmann (14) assumed at first that pyrophosphate occurred in muscle in a free state but when Davenport and Sacks (15) showed that a fresh trichloroacetic acid extract of muscle gave no color reaction for pyrophosphate, he suggested that it might be bound to some other compound. Soon afterwards Lohmann (16) recognized that this acid-labile fraction is in combination with adenylic acid, a compound which had been isolated from muscle by Embden and Zimmermann (17). Independently Fiske and Subbarow (18) reported the isolation of adenosinetriphosphate from muscle and they pointed out that this was the mother substance of Lohmann's pyrophosphate and Embden's adenylic acid.

phosphocreatine P (0 minute value) and total P were determined before and after incubation, and in addition samples were hydrolyzed for 7 minutes and for 4 hours at 100° in N HCl.

TABLE III

Influence of Autolysis on Hexosephosphate and Other P Compounds of Muscle

Minced muscle was incubated for 2 hours at 37° in 1 per cent sodium bicarbonate solution.

All values are given in mg. per 100 gm. of muscle.

Experiment No.	Hexosephosphate			Inorganic P after hydrolysis in N HCl			Total acid-soluble P	Remarks
	Hexose found	P found	P calculated	0 min.	7 min.	240 min.		
Before incubation								
1	121	20.8	20.8	108	152	174	201	
2	106	17.9	18.3	97	139	164	187	
3	107	18.6	18.5	120	163	184	206	
4	106	19.9	18.3	120	156	182	207	
Average..	110	19.3	19.0	111	152	176	200	
					+41	+24	+24	
After incubation								
1	76	12.6	13.1	183	187	195	208	
2	82	13.9	14.1	171	172	183	195	
2-a	88	16.1	15.2	170	173	182	197	Incubated for 4 hrs.
3	88	14.6	15.2	173	174	185	195	
3-a	91	16.3	15.7	187	195	203	214	Embden ester added*
4	89	11.8	15.3	182	185	192	203	
4-a	83	12.0	14.3	190	191	201	211	Embden ester added*
Average..	84	13.2	14.4	177	179	189	200	Experiments 2-a, 3-a, and 4-a not included in average
					+2	+10	+11	

The figures below the averages represent the difference between the averages for 7 and 0 minutes, etc.

* Corresponding to 10 mg. of P and 58 mg. of hexose per 100 gm. of muscle.

For an interpretation of the values in Table III it is necessary to mention that two-thirds of the P of the adenosinetriphosphate (the

pyrophosphate fraction of Lohmann) is split off during 7 minutes of hydrolysis in N HCl, leaving adenosinemonophosphate which gives off two-thirds of its P during 4 hours of hydrolysis at 100° in N HCl.[7] Hexosemonophosphate (Embden ester) is hydrolyzed with still greater difficulty than adenylic acid, since only 28 per cent or little more than one-fourth of its P is split off during 4 hours of hydrolysis.

Considering first the values before incubation in Table III, one finds that $152 - 111 = 41$ mg. of P were split off during 7 minutes hydrolysis in N HCl. From this an adenosinetriphosphate P of 61.5 mg. may be calculated. Since the hexosephosphate P was 19 mg.[8] a total of $61.5 + 19 = 80.5$ mg. of organically bound P is accounted for, while 89 mg. is the value for organically bound P calculated from the difference of total (200) and inorganic plus phosphocreatine (111) P in Table III. Hence there remain 8.5 mg. of unidentified, organically bound P.

It was thought at first that these 8.5 mg. might represent some adenylic or inosinic acid formed from adenosinetriphosphate either while the animal was being killed or during the process of mincing the muscle. Such a splitting of adenosinetriphosphate would decrease the 7 minute hydrolysis value and increase the inorganic P correspondingly. When muscle was removed from the living animal in the manner described in a preceding section, the average of twelve experiments for the 7 minute hydrolysis value was 44 and for inorganic plus phosphocreatine P 109 mg., which differs but little from the average values of 41 and 111 mg. reported in Table III. The unidentified P does not seem to be hexosediphosphate (Harden-Young ester) because 95 per cent of the latter is split during 4 hours of hydrolysis, while it may be calculated from the experiments in Table III that only 55 per cent of the unidentified P was hydrolyzed during 4 hours. There is the possibility that the unidentified P represents an as yet unknown P compound in muscle.

After incubation, the inorganic P was 66 mg. higher than before

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[7] This was determined on a sample of adenosinetriphosphate which Dr. Fiske was kind enough to send us. Since adenine is split off quite rapidly, it is really ribosephosphate which is undergoing hydrolysis.

[8] It should be noted that killing the animal before removal of muscle, as was done in these experiments, more than doubles the hexosephosphate content.

incubation (Table III). According to Embden's old terminology the "lactacidogen" P content of this muscle would have been 66 mg. Since the hexosephosphate content diminished on an average from 19 to 14.4 mg. during incubation, only a small fraction (less than 7 per cent) of the amount of inorganic P formed during autolysis came from this compound. While the 7 minute hydrolysis value was 41 mg. before, it was only 2 mg. after incubation. Hence this fraction which Lohmann designates as "pyrophosphate" contributed 39 mg. or 59 per cent of the inorganic P split off during autolysis. The adenylic or inosinic acid formed by partial dephosphorylation of adenosinetriphosphate disappeared almost completely and contributed close to one-third of the inorganic P liberated during autolysis. Calculated on the basis of adenosinetriphosphate, this compound was the source of about 90 per cent of the inorganic P formed during autolysis of muscle.

Before incubation there were 89 mg. of organically bound P present. After incubation there remained $200 - 177 = 23$ mg. of organically bound P which had resisted dephosphorylation. Deducting from this the hexosephosphate P of 14.4 mg., one finds 8.6 mg. of P. Part of this is probably adenylic (or inosinic) acid P.

The finding of Lohmann that the P liberated during autolysis of muscle is mostly "pyrophosphate" P is confirmed. But while Lohmann assumes that the hexosephosphate present in muscle is completely split during autolysis and contributes the remaining portion of the P liberated during autolysis, our direct analysis shows that this is not the case and that it is mainly adenylic acid derived from adenosinetriphosphate which is split under these conditions.

As mentioned before, Lohmann found that hexosephosphate added to minced muscle is completely split during autolysis. Since it was difficult to see why added hexosephosphate should behave differently from that originally present in muscle, it was decided to repeat Lohmann's experiment. In two cases Embden ester was added (Table III), and while the muscle hexosephosphate originally present diminished only 16.8 and 15.7 per cent, practically all of the added ester was split during 2 hours of incubation, in confirmation of Lohmann's results. It will not be attempted at the present time to give an explanation for this unusual result. That the alkaline reaction is not responsible for the disappearance

of added hexosephosphate was shown in an experiment in which the muscle hash was first made acid to Congo paper by means of trichloroacetic acid. After 5 minutes standing the pH was adjusted to 8.0 by means of sodium carbonate, Embden ester was added, and the sample kept for 2 hours at 37°. There was practically no disappearance of hexosephosphate during incubation presumably because the phosphatase had been destroyed by the treatment with acid.

In one experiment in Table III the same muscle hash was incubated for 2 and 4 hours respectively, but no additional hexosephosphate was split after 2 hours nor was there any further increment in the inorganic P. Embden had shown previously that the autolysis of phosphorus compounds in muscle practically comes to an end in 2 hours.

SUMMARY

1. A method for the simultaneous determination of hexosemonophosphate as hexose and as P in 1.5 to 2 gm. of muscle is described. The trichloroacetic acid extract of muscle is adjusted to pH 8.2 by means of barium hydroxide and after removal of the insoluble barium salts by centrifugation and recovery of some hexosephosphate which is included in the barium precipitate, the compound is precipitated in 80 per cent alcohol. The reducing power and the P content of the dissolved alcoholic precipitate are then determined. Embden ester added to the trichloroacetic acid filtrate is recovered satisfactorily.

2. A study was made of the autolysis of phosphorus compounds during incubation of minced muscle in sodium bicarbonate solution at 37°. Before incubation there were present per 100 gm. of muscle 62 mg. of adenosinetriphosphate P, 19 mg. of hexosephosphate P, and 8 mg. of unidentified P. Of the 66 mg. of inorganic P liberated during 2 hours of incubation, 61 mg. or 92.4 per cent came from adenosinetriphosphate and the undetermined P fraction. Only 15 to 30 per cent of the hexosephosphate present in muscle disappeared during autolysis, even if the incubation was extended over 4 hours; this compound contributed only 7 per cent of the inorganic P liberated during autolysis. Embden ester added to minced muscle was however completely split during 2 hours of incubation.

BIBLIOGRAPHY

1. Cori, C. F., and Cori, G. T., *Proc. Soc. Exp. Biol. and Med.*, **27**, 934 (1929–30).
2. Embden, G., and Zimmermann, M., *Z. physiol. Chem.*, **167**, 114 (1927).
3. Embden, G., and Zimmermann, M., *Z. physiol. Chem.*, **141**, 225 (1924).
4. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **94**, 581 (1931).
5. Lohmann, K., in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 2nd edition, suppl., 133 (1930).
6. Meyerhof, O., Die chemischen Vorgänge im Muskel, Berlin (1930).
7. Embden, G., Schmitz, E., and Meincke, P., *Z. physiol. Chem.*, **113**, 10 (1921).
8. Embden, G., and Jost, H., *Z. physiol. Chem.*, **179**, 24 (1928).
9. Eggleton, G. P., and Eggleton, P., *J. Physiol.*, **68**, 193 (1929–30).
10. Benedict, S. R., *J. Biol. Chem.*, **76**, 457 (1928).
11. Somogyi, M., *Proc. Soc. Exp. Biol. and Med.*, **26**, 353 (1928–29).
12. Michaelis, L., *Biochem. Z.*, **234**, 139 (1931).
13. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 376 (1925).
14. Lohmann, K., *Biochem. Z.*, **202**, 466 (1928).
15. Davenport, H. A., and Sacks, J., *J. Biol. Chem.*, **81**, 469 (1929).
16. Lohmann, K., *Naturwissenschaften*, **17**, 624 (1929).
17. Embden, G., and Zimmermann, M., *Z. physiol. Chem.*, **167**, 137 (1927).
18. Fiske, C. H., and Subbarow, Y., *Science*, **70**, 381 (1929).
19. Lohmann, K., *Biochem. Z.*, **203**, 172 (1928).

THE INFLUENCE OF EPINEPHRINE AND INSULIN INJECTIONS ON HEXOSEPHOSPHATE CONTENT OF MUSCLE

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It is a well established fact that injections of insulin in normal and diabetic men and animals cause a decrease in the inorganic phosphate content of the blood and a diminution in urinary phosphate excretion (Harrop and Benedict (1), Wigglesworth and collaborators (2), and others). As the effect of the insulin injection wears off, a compensatory increase in phosphate excretion occurs, so that the total excretion for 24 hours is generally not much altered. Carbohydrate ingestion is also followed by a temporary diminution in phosphate excretion in normal individuals, an observation made by Fiske (3) prior to the discovery of insulin. In diabetic dogs glucose ingestion does not lead to changes in urinary phosphate excretion (Bolliger and Hartman (4), Markowitz (5)).

The decrease in phosphate excretion has frequently been explained by an accumulation of hexosephosphate in the tissues and in fact several authors (1, 6, 7) claimed to have found an increase in the "lactacidogen" content of muscle after insulin injection, but other workers (8, 9), who used the same technique, were unable to verify this result. Besides, as shown in the preceding paper (10), Embden's old lactacidogen method which was used by these investigators does not determine hexosephosphate.

The observation of Perlzweig *et al.* (11), that epinephrine injections produce the same effect on blood phosphates as insulin injections, has offered considerable difficulties, because in other respects epinephrine has been found to be an antagonist of insulin. The changes in urinary phosphate excretion after epinephrine injection were studied particularly by Allan and collaborators (12)

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and were found to be of the same nature as those following an insulin injection.

As mentioned previously, the starting point for the present investigation was the observation that only part of the muscle glycogen which disappeared under the influence of epinephrine was immediately converted to lactic acid. Thus in the rat, 30 minutes after epinephrine injection, lactic acid formation corresponded to only 40 per cent of the glycogen lost from muscle (13). It became clear at once that here were especially favorable conditions for a demonstration of an intermediary of the glycogen → lactic acid transformation in the tissues. Preliminary experiments in which the recent method of Embden and Jost (14) was used made it seem probable that there occurred an accumulation of hexosephosphate in muscle during an early period of epinephrine

TABLE I

Hexosephosphate Content of Rat Gastrocnemius

The values are expressed in mg. per 100 gm. of muscle.

Experiment No.	1*	2	3	4	5	6	7	8*	9	Average
Hexose found	46.3	48.5	48.7	48.8	53.5	54.7	56.4	60.3	62.9	53.3
P found	9.3	7.4	8.2	8.9	8.4	7.8	8.0	13.2	9.1	8.9
" calculated	8.0	8.4	8.4	8 4	9.2	9.4	9.7	10 4	10.8	9.2

* Muscle taken from adrenalectomized animal.

action. The desire to verify this finding by means of a more reliable and specific method and interest in the rôle of hexosephosphate in carbohydrate metabolism of muscle in general led to the experiments described in the present two papers.

Hexosephosphate Content of Resting Muscle—The values shown in Tables I and III to VI are for muscle removed from amytalized rats in the manner described in the preceding paper (10). Since procedures which increase the lactic acid content of muscle, also cause a rise in hexosephosphate content, it is of importance to mention that lactic acid determinations in rat muscle removed under conditions identical to those obtaining in the experiments in Table I, showed resting values (10 to 25 mg. of lactic acid per 100 gm. of muscle). Separate hexosephosphate determinations in the right and left gastrocnemius of the same rat were made on

several occasions and closely agreeing values were obtained. The hexosephosphate content of various thigh muscles of the rat was very similar to that of the gastrocnemius. Amytal anesthesia of $\frac{1}{2}$ to 1 hour duration had no demonstrable influence on the hexosephosphate content of muscle. In rabbits it was found difficult to obtain resting values for hexosephosphate. Amytal is an unsatisfactory anesthetic for this species and urethane could not be used because it invariably caused an increase in hexosephosphate. Besides, rabbit muscle twitches much more than rat muscle when exposed and while it is excised. The lowest values obtained on rabbits were within the range of the values found for rat muscle.

The fact that hexosemonophosphate, the supposed intermediary of the glycogen → lactic acid transformation, is always present in considerable amounts in normal resting muscle, argues in favor of Meyerhof's (15) conception that the Embden ester is a stabilization product of another, very labile ester. The following scheme which may also serve as an explanation of some of the findings given below, illustrates this.

Glycogen
↓
Labile ester → Embden ester
↓
Lactic acid ←
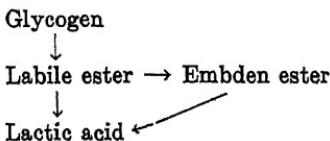
According to this scheme the labile ester does not exist for any length of time in the tissues; it is transformed *in statu nascendi* to lactic acid. That part which escapes this transformation is immediately converted into the stable Embden ester which forms lactic acid at a much slower rate. Under these conditions an increase in the speed of the side reaction, *i.e.* the formation of stable ester, might be expected to occur either if the rate of breakdown of glycogen is increased or if there is an inhibition of lactic acid formation. Examples for the former case are given in this paper, while the latter case is realized during poisoning with fluoride or iodoacetic acid.

Influence of Killing an Animal on Hexosephosphate Content— When an animal is killed by stunning, decapitation, bleeding, etc., before the muscle is taken for analysis, the hexosephosphate content rises to very high values, as shown in Table II. This

accumulation of hexosephosphate (and of lactic acid) is due to intense nervous discharges and cannot be prevented even if the greatest possible speed is used in the removal of muscle after

TABLE II

Hexosephosphate Content of Muscle Taken Immediately after Death

In Experiments 1 and 2 the animals were bled to death under amytal and in Experiments 3 to 5 they were killed by decapitation.

The values are expressed in mg. per 100 gm. of muscle.

Experiment No.	1	2	3	4	5
Hexose found	110	121	140	185	219
P found	18.7	20.8	22 6	32.5	37.2
" calculated	19.0	20.9	24.2	31.9	37.8

TABLE III

Influence of Epinephrine on Hexosephosphate Content of Muscle

0.02 mg. of epinephrine per 100 gm. of body weight was injected subcutaneously.

The values are expressed in mg. per 100 gm. of muscle.

Experiment No. Min. after injection		1 15	2 15	3* 30	4* 30	5 35	6 40	7 40	8 45	9 47	10 48	11 50
	0											
Hexose found	53.3	87.0	89.1	83.5	90.4	100.2	81.3	92.6	106.6	91.2	119.0	116.0
P found	8.9	14.8	12.9	14.7	14.9	17.2	14.0	15.8	16.4	15.0	18.2	20.3
" calculated	9.2	15.0	15.3	14.4	15.6	17.3	14.5	16.0	18.3	15 7	20.5	20.0
Average (as hexose)	53.3	88.0		86.9		91.3			108.2			

Experiment No. Min. after injection	12 60	13 60	14 90	15 120	16 120	17 180	18 180	19 240
Hexose found	101.4	115.8	83.3	71.4	73.6	63.2	69.4	54.0
P found	16.2	19.0	15.1	13.6	11.8	11.4	12.5	8.2
" calculated	17.5	19.9	14.3	12.3	12.7	10.9	12.0	9.4
Average (as hexose)	108.6		83.3		72.8		66.3	54.0

* Epinephrine was injected intravenously at a rate of 0.001 mg. per kilo per minute.

stunning, because less than 5 seconds of tetanic stimulation are sufficient to cause a marked rise in hexosephosphate. Bleeding an anesthetized animal to death also causes an increase in the hexosephosphate content of muscle.

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It is still a quite common practice to kill an animal before muscle is removed for glycogen analysis, though the objections to such a procedure have frequently been pointed out. Since the hexosephosphate content (in terms of hexose) may rise 0.1 per cent or more and the lactic acid content of muscle after killing is generally in the neighborhood of 0.2 per cent, the total loss of glycogen under these conditions may be as high as 0.3 per cent.

Influence of Epinephrine Injections—A determination of the blood lactic acid curve in rats after subcutaneous injection of epinephrine was made on a previous occasion (16). 15 minutes after injection of 0.2 mg. per kilo the blood lactic acid was doubled; it reached its peak in 1 hour and then slowly returned to the basal level in the next 2 hours. Since hexosephosphate is regarded as the precursor of lactic acid, it was of interest to study the relation between the hexosephosphate content of muscle and the blood lactic acid curve. Table III shows that the muscle hexosephosphate is definitely increased 15 minutes after the epinephrine injection, reaches the highest value after 1 hour, and then returns to the original level in the next 2 to 3 hours; *i.e.*, it runs almost exactly parallel to the blood lactic acid curve. With the demonstration of a decrease in glycogen, increase in lactic acid, and accumulation of hexosephosphate as the intermediary of this transformation, the glycogenolytic effect of epinephrine in muscle may be regarded as well established.

When an increase in hexosephosphate of the same magnitude as that occurring after epinephrine injection is produced by electrical stimulation of muscle, it takes about 10 minutes until the hexosephosphate content returns to the normal level. The much longer period of time during which the hexosephosphate content remains elevated after subcutaneous injection of epinephrine and the slow return to the basal level indicate that new formation of hexosephosphate persists for some time after the injection. This is in accord with previous observations of a prolonged effect of subcutaneously injected epinephrine on carbohydrate metabolism and finds its explanation in slow absorption of epinephrine from the subcutaneous tissue.

Muscular activity can be excluded as the cause of the increase in muscle hexosephosphate after epinephrine injection, because an increase is also observed when the injected animals are kept under

Hydrolysis of Trichloroacetic Acid Extract of Muscle in N HCl at 100°

The P values are expressed in mg. per 100 gm. of muscle; the sugar values in mg. per 100 cc. of plasma.

Table No.	Experiment No.	Inorganic P after				Total acid-soluble P	Sugar in arterial plasma	Remarks
		0 min.*	7 min.	30 min.	180 min.			
I	1	117	161	168	178	200	92	Control
	2	109	157	158	169	185	108	
	4	115	150	156	169	191	139	
	6	112	154	158	167	192		
	9	108	157	161	178	196	120	
Average.....		112	156	160	172	193	115	
			+44	+4	+12	+21		
III	1	99	154	156	170	181	158	Epinephrine
	10	106	152	155	168	195	216	
	11	84	138	143	154	183	361	
	12	96	136	141	154	177	236	
	14	121	161	167	179	202	309	
	15	107	151	159	170	204	219	
	17	107	152	161	171	194		
	18	103	146	153	169	187		
Average.....		103	149	154	167	190		
			+46	+5	+13	+23		
V	1	95	140	145	156	181	54	Insulin
	2	100	144	148	158	186	52	
	5	105	151	154	166	192	50	
Average.....		100	145	149	160	186	52	
			+45	+4	+11	+26		
V	6	117	165	173	183	208	92	Glucose plus insulin
	7	101	148	152	165	177	98	
	8	100	146	153	175	189	64	
	9	99	139	147	163	182	71	
Average.....		104	149	156	171	189	81	
			+45	+7	+15	+18		
V	10	109	156	165	173	190	29	Insulin after adrenalectomy
	12	107	151	156	169	185	22	
	13	118	162	168	178	195	38	
	14	100	143	149	159	183	29	
Average.....		108	153	159	170	188	29	
			+45	+6	+11	+20		

The figures below the averages represent the difference between the averages for 7 and 0 minutes, etc.

amytal anesthesia in a state of complete muscular relaxation. Nor can the dose of epinephrine be held responsible, since an intravenous injection at a rate of 0.001 mg. per kilo per minute for 30 minutes, which is within physiological limits, also produced an increase in the hexosephosphate content of muscle (Table III).

Meyerhof (15) found that apart from hexosemono- a large amount of hexosediphosphate (of the Harden-Young type) was formed from added glycogen by a glycolyzing, cell-free muscle extract. Hydrolysis curves in N HCl at 100° gave no evidence for the accumulation of a hexosediphosphate in intact muscle during epinephrine action. It will be noted in Table IV that the trichloroacetic acid extract of normal muscle and of muscle taken from animals injected with epinephrine was hydrolyzed for 7, 30, and 180 minutes. The rate at which various phosphorus compounds are hydrolyzed under these conditions, was given in the preceding paper. It may be added that Harden-Young ester yields 55 per cent of its P during 30 minutes and 90 per cent during 180 minutes of hydrolysis. Hence, the amount of P split off between 7 and 180 minutes of hydrolysis should become larger if Harden-Young ester were formed during epinephrine action. Such was not the case, as may be seen by a comparison of the average values in Table IV.

During muscular contraction phosphocreatine is split and part of the phosphate thus liberated is used for the formation of hexosephosphate. No appreciable change in the phosphocreatine content of muscle could be detected when small doses of epinephrine were injected (17). Nor is any P split off from adenosinetriphosphate under the influence of epinephrine. This is shown by the fact that the average amount of P liberated during 7 minutes of hydrolysis is practically the same for the control animals and for the animals injected with epinephrine (Table IV). Since previous experiments (17) have shown a slight decrease in the inorganic phosphate content of muscle, it is probably phosphate from this fraction which is used for hexosephosphate formation during epinephrine action. The decrease in inorganic phosphates in blood and urine observed by others after epinephrine injection is thus accounted for by a temporary accumulation of hexosephosphate throughout the muscles of the body.

Influence of Insulin Injections—In the experiments in Table V

2 units of insulin were injected into rats fasted previously for 24 hours. The plasma sugar was determined in the Folin-Wu filtrate with the Hagedorn-Jensen method. 60 to 90 minutes after the injection a pronounced hypoglycemia had developed. The muscle was taken for analysis before the onset of convulsions. Its hexosephosphate content had risen to twice the normal value. This result seemed at first very puzzling because it was difficult to see how insulin and epinephrine with their opposite effect on muscle glycogen could have the same effect on hexosephosphate.

TABLE V

Influence of Insulin on Hexosephosphate Content of Muscle

The muscle was obtained 60 to 90 minutes after injection.
For blood sugar values see Table IV.
The values are expressed in mg. per 100 gm. of muscle.

Experiment No............	1	2	3	4	5	Average	
Hexose found........	96.3	99.0	103.3	117.0	119.7	107.0	Insulin injected in
P found.............	17.1	16.4	16.5	20.2	20.0	18.0	fasted rats
" calculated........	16.6	17.0	17.8	20.1	20.8	18.4	
Experiment No............	6	7	8	9			
Hexose found........	57.0	58.5	64.8	71.8		63.0	Insulin injected
P found.............	9.7	9.5	11.9	12.7		10.9	during glucose
" calculated........	9.8	10.0	11.2	12.4		10.9	absorption
Experiment No............	10	11	12	13	14		
Hexose found........	41.6	42.4	44.4	56.7	59.4	48.9	Insulin injected in
P found.............	7.8	7.2	7.8	8.5	11.0	8.4	fasted, adrenal-
" calculated........	7.2	7.3	7.6	9.7	10.2	8.5	ectomized rats

There was the possibility of a chemical difference in the esters formed during insulin and epinephrine action, but the rate of hydrolysis of the two esters in N HCl proved to be the same. Experiments in which insulin was injected into rats during glucose absorption brought the desired explanation. The hexosephosphate content of muscle did not rise (Experiments 6 and 7, Table V) or rose only slightly (Experiments 8 and 9, Table V), depending on the degree to which the hypoglycemia was prevented by the glucose feeding. The next step was to see whether the hypoglycemia had a direct effect on muscle, leading to an increase in hexose-

phosphate, or whether the effect was an indirect one, mediated by the adrenals. Experiments 10 to 14 in Table V show that insulin injected into adrenalectomized animals does not produce a rise in the hexosephosphate content of muscle, in spite of a severe hypoglycemia (average arterial plasma sugar 29 mg. per cent). It may therefore be concluded that the increase in hexosephosphate observed in normal animals after insulin injection is due to a reflex discharge of epinephrine elicited by the hypoglycemia. The occurrence of an increased discharge of epinephrine during insulin hypoglycemia has been established by the work of Cannon, McIver, and Bliss (18) and others.

It remains to be investigated whether the decrease in inorganic phosphate in blood and urine after insulin injection is due entirely

TABLE VI

Influence of Glucose Feeding on Hexosephosphate Content of Muscle

The muscle was obtained 60 to 120 minutes after the feeding while active absorption was still going on.

The values are expressed in mg. per 100 gm. of muscle.

Hexose found	49.3	50.5	53.5	64.5	54.4 average
P found	10.0	8.2	9.3	8.7	9.0 "
" calculated	8.5	8 7	9.2	11.1	9.2 "

to the increase in hexosephosphate content of the muscles brought about by the secondary output of epinephrine.

Effect of Glucose Administration—Glucose feeding does not lead to changes in the hexosephosphate content of muscle (Table VI). The diminution of blood and urinary phosphate after a carbohydrate meal remains thus unexplained; *i.e.*, all that can be said about it at the present time is that this diminution is not due to accumulation of hexosephosphate. Since glucose feeding is followed by glycogen formation in muscle, it would seem as if hexosephosphate were not an intermediary in the synthesis of glucose to glycogen.

SUMMARY

1. The average hexosephosphate content of muscle removed from the living animal under amytal was found to be 53.3 mg. per cent as hexose and 8.9 mg. per cent as P, while the P calculated

from the hexose was 9.2 mg.   When the hexosephosphate content of muscle was increased by means of the various procedures described below, the agreement between P determined and P calculated from the hexose content was equally satisfactory.

2. When muscle is taken immediately after killing an animal, the hexosephosphate content of muscle is always markedly increased and may be as high as 200 mg. per cent (as hexose).

3. Following subcutaneous injection of epinephrine the hexosephosphate content of muscle is increased after 15 minutes, reaches its peak after 1 hour (109 mg. per cent as hexose), and slowly returns to the basal level 4 hours after the injection.

4. Insulin injections in fasted rats are followed by an increase in hexosephosphate.   When the hypoglycemia is prevented by simultaneous administration of glucose or when insulin is injected into adrenalectomized animals, producing a severe hypoglycemia, the hexosephosphate content of muscle remains unchanged. Hence, it is a secondary output of epinephrine which is responsible for the increase in hexosephosphate during insulin hypoglycemia.

5. Hydrolysis curves of the muscle extract in N HCl gave practically identical values under the different experimental conditions mentioned.   There was no indication of an accumulation of hexosediphosphate (of the Harden-Young type) during insulin or epinephrine action, nor was any P split off from adenosinetriphosphate.

6. Glucose feeding did not lead to changes in the hexosephosphate content of muscle.   While the temporary decrease in blood and urinary phosphate after epinephrine or insulin injection might be due to the accumulation of hexosephosphate in muscle, this explanation does not hold for the decrease in phosphate excretion observed after a carbohydrate meal.

BIBLIOGRAPHY

1. Harrop, G. A., Jr., and Benedict, E. M., *Proc. Soc. Exp. Biol. and Med.*, 20, 430 (1923); *J. Biol. Chem.*, 59, 683 (1924).
2. Wigglesworth, V. B., Woodrow, C. E., Smith, W., and Winter, L. B., *J. Physiol.*, 57, 447 (1922–23).
3. Fiske, C. H., *J. Biol. Chem.*, 41, p. lix (1920).
4. Bolliger, H., and Hartman, F. W., *J. Biol. Chem.*, 64, 91 (1925).
5. Markowitz, J., *Am. J. Physiol.*, 76, 525 (1926).
6. Audova, A., and Wagner, R., *Klin. Woch.*, 3, 231 (1924).

7. Kay, H. D., and Robison, R., *Biochem. J.*, **18**, 1139 (1924).
8. Macleod, J. J. R., Carbohydrate metabolism and insulin, London and New York (1926).
9. Best, C. H., Hoet, J. P., and Marks, H. P., *Proc. Roy. Soc. London, Series B*, **100**, 32 (1926).
10. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **94,** 561 (1931).
11. Perlzweig, W. A., Latham, E., and Keefer, C. S., *Proc. Soc. Exp. Biol. and Med.*, **21**, 33 (1923–24).
12. Allan, F. N., Dickson, B. R., and Markowitz, J., *Am. J. Physiol.*, **70**, 333 (1924).
13. Cori, C. F., and Cori, G. T., *Proc. Soc. Exp. Biol. and Med.*, **27**, 934 (1929–30).
14. Embden, G., and Jost, H., *Z. physiol. Chem.*, **179**, 24 (1928).
15. Meyerhof, O., Die chemischen Vorgänge im Muskel, Berlin (1930).
16. Cori, G. T., Cori, C. F., and Buchwald, K. W., *J. Biol. Chem.*, **86**, 375 (1930).
17. Cori, G. T., *Am. J. Physiol.*, **94**, 557 (1930).
18. Cannon, W. B., McIver, M. A., and Bliss, S. W., *Am. J. Physiol.*, **69**, 46 (1924).

CONFIGURATIONAL RELATIONSHIP OF α-HYDROXY-HEPTANOIC ACID TO OTHER α-HYDROXY ACIDS

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In the course of the investigations of Levene and Haller,[1] dealing with the problem of the relationship of chemical structure to optical activity, it was found that α-hydroxy acids of the con-

figuration COOH—$\overset{\displaystyle OH}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C}}}}$—R rotated to the left. Up to the date of the

present communication, α-hydroxycaproic acid was the highest member of the series which had been investigated. If the rotations of these acids are compared with those of the secondary carbinols, it will be found that the carbinols rotating in the

same direction have the configuration C_2H_5—$\overset{\displaystyle OH}{\underset{\displaystyle R}{\overset{|}{\underset{|}{C}}}}$—$CH_3$. In other

words in the series of α-hydroxy acids thus far examined, the carboxyl group functions as the heavier group as compared with the alkyl group although the relative weight of the carboxyl group is only 45 as compared with 57, the weight of the butyl group. The question naturally arises whether the same relationship of structure to rotation of α-hydroxy acids will hold in those cases in which the alkyl group attached to the asymmetric carbon atom has a still higher weight. Because of these considerations, it was desired to correlate the configuration of α-hydroxyheptanoic acid with that of the α-hydroxy acids previously prepared. The

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[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

relationship has been established through the intermediary of the unsaturated carbinol, a method which had been employed with success in previous investigations.[1]   The procedure is outlined by the following set of transformations.

$$
\begin{array}{ccccc}
 & & & & \mathrm{CH_2OH} \\
 & & & & | \\
 & & & & \mathrm{HCOH} \\
 & & & & | \\
 & & & & \mathrm{C_5H_{11}} \\
\mathrm{CH_3} & \mathrm{CH_2} & & & \textit{Dextro} \\
| & || & & & \\
\mathrm{CH_2} & \mathrm{CH} & \mathrm{HC{=}O} & & \\
| & | & | & & \\
\mathrm{HCOH} \leftarrow & \mathrm{HCOH} \rightarrow & \mathrm{H{-}C{-}OH} & & \\
| & | & | & & \\
\mathrm{C_5H_{11}} & \mathrm{C_5H_{11}} & \mathrm{C_5H_{11}} & & \\
\textit{Dextro} & \textit{Levo} & \textit{Dextro} & & \\
 & & & & \mathrm{COOH} \\
 & & & & \\
 & & & & \mathrm{HCOH} \\
 & & & & | \\
 & & & & \mathrm{C_5H_{11}} \\
 & & & & \textit{Levo}
\end{array}
$$
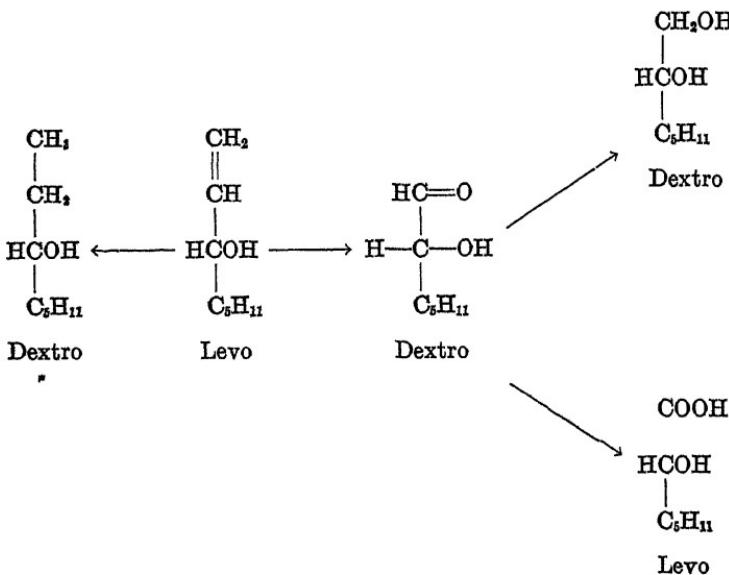
Thus, the carboxyl group, with a relative weight of 45, functions as a group heavier than the normal amyl radicle, weighing 71.

The sodium salt of this acid was dextrorotatory, thus showing that on passing to the ionized state the change of rotation of this acid is to the right as in the lower members of the hydroxy acids of this series.

<div align="center">EXPERIMENTAL</div>

<div align="center"><i>Relation between Amylvinylcarbinol and Amylethylcarbinol</i></div>

Amylvinylcarbinol—This carbinol was obtained by the condensation of molecular proportions of acrolein and amyl magnesium bromide in the usual manner.   It distilled at 81–82° at 20 mm.

Resolution of Amylvinylcarbinol—A mixture of 215 gm. of amylvinylcarbinol, 275 gm. of phthalic anhydride, and 350 cc. of dry pyridine was allowed to stand overnight and was then heated on the steam bath for 60 minutes.   The isolation and purification

of the acid phthalate was effected in the usual way.[2] It was recrystallized from benzene and melted at 76–79°. The acid phthalate was dissolved in acetone and was readily converted into the strychnine salt. It was extracted with a large volume of acetone. The insoluble salt was once recrystallized from 90 per cent alcohol. The usual procedure was employed for the isolation of the carbinol. It distilled at 78° at 19 mm. and had the following composition.

4.171 mg. substance: 11.490 mg. CO_2 and 4.760 mg. H_2O.
$C_8H_{16}O$ (128.1). Calculated. C 74.94, H 12.59
Found. " 75.13, " 12.77

The rotation without solvent was $\alpha_D^{24} = -17.1°$. The specific rotation of levo-amylvinylcarbinol was

$$[\alpha]_D^{27} = \frac{-1.60° \times 100}{1 \times 8.68} = -18.43° \text{ (in absolute alcohol)}$$

$$[\alpha]_D^{27} = \frac{-1.15° \times 100}{1 \times 7.89} = -14.58° \text{ (in heptane)}$$

Dextro-Amylvinylcarbinol—This dextrorotatory carbinol was obtained from the mother liquors of the acetone-insoluble strychnine salt just described. Its rotation without solvent was $\alpha_D^{25} = +14.3°$.

Reduction of Dextro-Amylvinylcarbinol to Levo-Amylethylcarbinol

Levo-Octanol-3—5 gm. of dextro-amylvinylcarbinol ($\alpha_D^{25} = +14.3°$) dissolved in 15 cc. of alcohol were reduced rapidly with hydrogen in the presence of platinum oxide catalyst after which the filtered solution was concentrated. The carbinol distilled at 82° at 24 mm. and had a rotation of $\alpha_D^{25} = -4.65°$ (homogeneous). It had the following composition.

2.825 mg. substance: 7.610 mg. CO_2 and 3.495 mg. H_2O.
$C_8H_{18}O$ (130.1). Calculated. C 73.79, H 13.94
Found. " 73.47, " 13.84

The specific rotation of the amylethylcarbinol was as follows:

$$[\alpha]_D^{27} \quad \frac{-0.75° \times 100}{1 \times 10.13} \quad -7.40° \text{ (in absolute ethyl alcohol)}$$

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **75**, 587 (1927).

A levorotatory amylvinylcarbinol of $\alpha_D^{25} = -7.7°$ (homogeneous) gave on reduction by the same method a dextrorotatory amyl-ethylcarbinol of $\alpha_D^{25} = +2.75°$ (homogeneous).

Urethane of Levo-Octanol-3—A mixture of 0.65 gm. of the above levo-amylethylcarbinol with 0.85 gm. of α-naphthylisocyanate was heated for 15 minutes at 100°. The crystalline material obtained on cooling was recrystallized from hot dilute alcohol. It melted at 79–80°. The composition of the substance was as follows:

5.919 mg. substance: 0.253 cc. N (29°, 762 mm.).
$C_{19}H_{25}O_2N$ (299.2). Calculated. N 4.68. Found. N 4.84

The specific rotation of the urethane was as follows:

$$[\alpha]_D^{30} \quad \frac{-0.15° \times 100}{1 \times 6.04} \quad -2.48° \text{ (in absolute ethyl alcohol)}$$

Relation between Amylvinylcarbinol and Heptandiol-(1, 2)

Dextro-α-Hydroxyheptanoic Aldehyde—A solution of levo-amyl-vinylcarbinol ($\alpha_D^{26} = -15.2°$) in glacial acetic acid was ozonized and the product isolated as previously described.[3] On fractiona-tion, a portion was received which distilled at 70–75° at about 3 mm., the receiving flask being kept in a cooling mixture. The aldehyde had the following composition.

3.570 mg. substance: 8.395 mg. CO_2 and 3.492 mg. H_2O.
$C_7H_{14}O_2$ (130.1). Calculated. C 64.57, H 10.84
                             Found.   " 64.13, " 10.94

The rotation of the substance rose from $\alpha_D^{25} = +5°$ to $\alpha_D^{25} = +27°$ within 1 hour. This change of rotation was probably due to dimerization.[3]

Reduction of Dextro-α-Hydroxyheptanoic Aldehyde to Dextro-Heptandiol-(1, 2)—A solution of 6.5 gm. of the freshly prepared dextro-α-hydroxyheptanoic aldehyde in 210 cc. of 80 per cent alcohol was stirred and sodium amalgam was added from time to time. 10 per cent sulfuric acid was added in such a manner as to keep the medium only slightly alkaline. When the mixture no longer reduced Fehling's solution it was completely neutralized and concentrated. The reaction product was extracted with a

[3] Levene, P. A., and Walti, A., **94**, 353 (1931).

mixture of absolute alcohol and ether and the solution dried over potassium carbonate. After removal of the solvents, 3.5 cc. of the glycol distilled at 90° at approximately 1 mm. The rotation of the substance was $\alpha_D^{24} = +2.25°$ (homogeneous). It had the following composition.

4.883 mg. substance: 11.400 mg. CO_2 and 5.145 mg. H_2O.
$C_7H_{16}O_2$ (132.1). Calculated. C 63.59, H 12.21
Found.   " 63.67, " 11.79

The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+1.25° \times 100}{1 \times 10.49} = +11.92° \text{ (in absolute ethyl alcohol)}$$

Diphenylurethane of Dextro-Heptandiol-(1, 2)—The urethane was prepared in the usual manner. It was recrystallized from dilute alcohol and melted at 109°. It had the following composition.

5.685 mg. substance: 0.396 cc. N (29.5°, at 760 mm.).
$C_{21}H_{26}O_4N_2$ (370.2). Calculated. N 7.57. Found. N 7.87

The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+0.29° \times 100}{1 \times 2.52} = +11.51° \text{ (in absolute ethyl alcohol)}$$

Oxidation of Dextro-α-Hydroxyhepanoic Aldehyde—The oxidation was performed by Goebel's method.[4] A solution of 6 gm. of dextro-α-hydroxyheptanoic aldehyde in 10 cc. of methyl alcohol was added to 650 cc. of Levene and Mikeska's Solution A[5] in the cold and then 910 cc. of the barium hydroxide Solution B was added in the course of 10 minutes with vigorous stirring at 0°. When all the barium hydroxide had been added, the reaction was allowed to proceed for 5 minutes. The product was then isolated in the usual manner. On concentration of the aqueous solution under diminished pressure, the barium salt of the hydroxy acid crystallized out in beautiful plates. The yield from 19.0 gm.

[4] Goebel, W. F., *J. Biol. Chem.*, **72**, 809 (1927).
[5] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **88**, 792 (1930).

of the hydroxy aldehyde was 5.0 gm. of the barium salt. The composition of the salt was as follows:

4.335 mg. substance: 6.213 mg. CO_2 and 2.375 mg. H_2O.
9.983 "   "   : 5.438 " $BaSO_4$.
$C_7H_{13}O_3\frac{1}{2}Ba$ (213.8). Calculated. C 39.29, H 6.13, Ba 32.12
                              Found.   " 39.09, " 6.13, " 32.05

Owing to the insolubility of the barium salt, the optical rotation of the sodium salt was observed. The greater part of the material was lost by accident. However, a small quantity was saved which was sufficient for the measurement of the rotation which was as follows:

$$[\alpha]_D^{25} = \frac{+\ 0.60° \times 100}{1 \times 8} = +\ 7.5° \ (\pm\ 0.02) \ (\text{in water})$$

The solution of the sodium salt was acidulated with sulfuric acid and extracted with ether. The ethereal solution was dried with sodium sulfate, filtered, and allowed to evaporate spontaneously in a desiccator under reduced pressure. The weight of the residue was 0.225 gm. It was dissolved in dilute alcohol and the following rotation was obtained.

$$[\alpha]_D^{25} = \frac{-\ 0.09° \times 100}{2 \times 4.5} = -\ 1.0° \ (\pm\ 0.005)$$

A NEW SYNTHESIS OF GLUTAMIC ACID*

By MAX S. DUNN, B. W. SMART, C. E. REDEMANN, AND K. E.
BROWN

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Angeles)*

Glutamic acid has been prepared previously by the racemization (1) of the natural dextro form and by three synthetic methods. The first synthesis, that by Wolff (2) in 1890, is principally of theoretical interest as it involves the preparation of six intermediate substances starting with levulinic acid and the yield of final product is not given. In 1925 Knoop and Oesterlin (3) obtained 1.1 gm. (23 per cent yield) of glutamic acid from α-ketoglutaric acid and alcoholic ammonia with hydrogen and palladium black as reducing agents. In the same year Keimatsu and Sugasawa (4) reported a synthesis with acrolein as starting material. None of these methods appears to be as satisfactory as the method described herewith.

The following reactions are involved in the present synthesis of glutamic acid.

$$
\begin{array}{ccccc}
\text{COOC}_2\text{H}_5 & & \text{COOC}_2\text{H}_5 & & \text{COOC}_2\text{H}_5 \\
| & & | & & | \\
\text{CH}_2 & \xrightarrow{\text{HNO}_2} & \text{CNOH} & \xrightarrow[\text{(Al—Hg)}]{\text{H}_2} & \text{CHNH}_2 & \xrightarrow[\text{(Na}_2\text{CO}_3)]{\text{C}_6\text{H}_5\text{COCl}} \\
| & & | & & | \\
\text{COOC}_2\text{H}_5 & & \text{COOC}_2\text{H}_5 & & \text{COOC}_2\text{H}_5 \\
\text{I} & & \text{II} & & \text{III}
\end{array}
$$

$$
\begin{array}{ccc}
\text{COOC}_2\text{H}_5 & & \text{COOC}_2\text{H}_5 \\
| & & | \\
\text{CHNHCOC}_6\text{H}_5 & \xrightarrow[\text{(C}_2\text{H}_5\text{ONa)}]{\text{BrCH}_2\text{CH}_2\text{COOC}_2\text{H}_5} & \text{C(CH}_2\text{CH}_2\text{COOC}_2\text{H}_5)\text{NHCOC}_6\text{H}_5 \\
| & & | \\
\text{COOC}_2\text{H}_5 & & \text{COOC}_2\text{H}_5 \\
\text{IV} & & \text{V}
\end{array}
$$

$$\xrightarrow[\text{(HCl)}]{4H_2O} \begin{array}{c} COOH \\ | \\ CHNH_2 \cdot HCl \\ | \\ CH_2CH_2COOH \end{array} + CO_2 + 3C_2H_5OH + C_6H_5COOH$$

VI

$$\xrightarrow{Ag_2O} \begin{array}{c} COOH \\ | \\ CHNH_2 \\ | \\ CH_2CH_2COOH \end{array}$$

VII

Method 1

$$\begin{array}{c} COOC_2H_5 \\ | \\ CHNH_2 \\ | \\ COOC_2H_5 \end{array} \xrightarrow[\text{(C}_2H_5ONa)]{ClCH_2CH_2COOC_2H_5} \begin{array}{c} COOC_2H_5 \\ | \\ C(CH_2CH_2COOC_2H_5)NH_2 \\ | \\ COOC_2H_5 \end{array} +$$

III   V-A

$$\begin{array}{c} COOC_2H_5 \\ | \\ CHNHCH_2CH_2COOC_2H_5 \\ | \\ COOC_2H_5 \end{array} \xrightarrow[\text{(HCl)}]{3H_2O} \begin{array}{c} COOH \\ | \\ CHNH_2 \cdot HCl \\ | \\ CH_2CH_2COOH \end{array} + CO_2 + 3C_2H_5OH +$$

V-B   VI

$$\begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ NHCH_2CH_2COOH \end{array} \xrightarrow{Ag_2O} \begin{array}{c} COOH \\ | \\ CHNH_2 \\ | \\ CH_2CH_2COOH \end{array}$$

VI-A   VII

Method 2

Isonitrosomalonic ester (II) was prepared from malonic ester and nitrous acid by the method of Cerchez (5).

The preparation of aminomalonic ester (III) presents some

unusual difficulties whether the reduction of isonitrosomalonic ester is carried out in acid, alkaline, or neutral medium.   In acid solution an amino derivative is formed, while the ester may be hydrolyzed by alkaline reduction.   If neutral solutions are used, an active metal or amalgam catalyst is required.

Since Johnson and Nicolet (6) obtained a 47 per cent yield of aminomalonic ester hydrochloride from the reduction of 10 gm. of isonitrosomalonic ester by hydrogen sulfide in alkaline solution, it seemed desirable to test this method with larger quantities of the isonitroso ester.   71.6 gm. (0.38 mol) of isonitrosomalonic ester and 22.1 gm. (0.38 mol plus 1 gm. excess) of potassium hydroxide were mixed with 400 cc. of water.   Aliquot portions of this mixture were withdrawn for analysis of amino nitrogen by the Van Slyke gasometric method.   It was shown that in 12 hours, the time used by Johnson and Nicolet, the reduction was 97 per cent complete.   As no data on the reaction of nitrous acid with aminomalonic ester could be found, the deaminization chamber was shaken for an arbitrarily chosen time of 10 minutes.

Calculated on the basis of the isonitrosomalonic ester taken, the final yield of aminomalonic ester hydrochloride was only 14.2 per cent of the theoretical, while it was 73.4 per cent based on the amino substances present in the combined ether fractions.   These findings confirm the statement of Johnson and Nicolet that with more than 10 to 20 gm. of isonitrosomalonic ester the percentage yield is decreased.   That this is due to the hydrolysis of the ester to aminomalonic acid was verified by isolating from the mother liquor 30.4 gm. of lead aminomalonate, which was converted to 10.2 gm. of crystalline aminomalonic acid (37 per cent of the theoretical amount).   The method of Baeyer (7) was used for isolating these substances.

Johnson and Nicolet state that aminomalonic ester hydrochloride, prepared in this manner, is practically pure, while Cerchez (8) found that it was hygroscopic and difficult to purify.   The product prepared in the present investigation was impure and contained nitrogen other than that in the amino form.   It is interesting to note that the reaction of aminomalonic ester with nitrous acid is not complete in 5 minutes.   Thus the behavior of this aminodicarboxylic acid resembles that of the aminomonocarboxylic acids (9) in which the amino group is present in other than the α position.

Calculated for $C_7H_{14}O_4NCl$. N 6.62. Found N (Kjeldahl) 6.37, 6.25, 6.25; (Van Slyke) 4.44 (5 min.), 4.84 (10 min.), 5.14 (30 min.), 5.10 (60 min.). Calculated. Cl 16.77. Found Cl (Volhard) 16.69, 16.72.

Catalytic methods for the reduction of isonitrosomalonic ester are generally unsatisfactory. After treating 5 gm. of isonitroso-malonic ester for 2 days with hydrogen and platinum black, Putochin (10) obtained a 60 per cent yield of aminomalonic ester hydrochloride. It was shown by Locquin and Cerchez (11) that hydrogen and nickel in an alcoholic medium did not reduce the ester in 8 hours at room temperature. In the present work an attempted reduction with hydrogen and platinum black gave negative results. Here the catalyst was prepared by the method of Adams, Voorhees, and Shriner (12) and its activity tested with maleic acid as described by Adams and Voorhees (13). The absorption of hydrogen was negligible in 3 hours, with a 25 gm. sample of isonitrosomalonic ester dissolved in 95 per cent ethyl alcohol, even though the catalyst was reactivated during this time and activator ions (ferrous and zinc) were added. The results were similar with ethyl acetate as solvent.

Locquin and Cerchez (11) were not able to effect reduction with magnesium amalgam but these authors, as well as Putochin (10) and Piloty and Neresheimer (14), found that aluminum amalgam is a satisfactory reducing agent. In the present investigations the reduction with aluminum amalgam went smoothly when the conditions established by Cerchez were followed. However, it is not easy to agree with Cerchez that aluminum is an effective agent only when in the form of platelets 20 to 25 mm. long, 6 to 8 mm. wide, and exactly 0.3 mm. thick. Since aluminum foil was used successfully in the present work, it is our opinion that the reducing power of aluminum amalgam depends on the purity of the metal, while the dimensions of the pieces should influence only the rate of the reduction.

The intermediate benzoylaminomalonic ester (IV) is a stable crystalline compound easily prepared in almost theoretical yield. The benzoyl radical not only fully protects the amino group from reaction with the halogen acid ester but it is easily removed from the resulting reaction product (V) by acid hydrolysis. When free aminomalonic ester was used by Keimatsu and Kato (15) for the synthesis of aspartic acid, a 33 per cent yield of iminodiacetic acid,
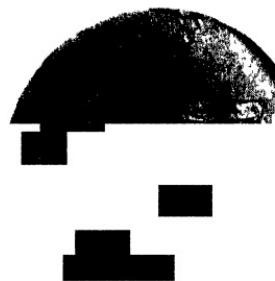
FIG. 1. Photomicrograph of *d*-glutamic acid. × 69.75
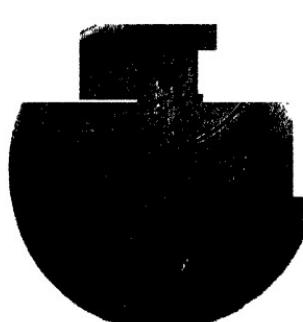
FIG. 2
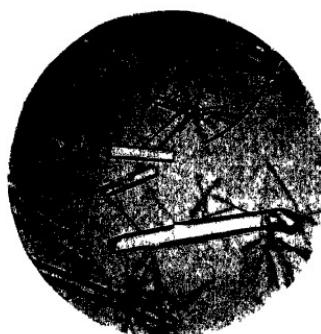
FIG. 3

FIG. 4
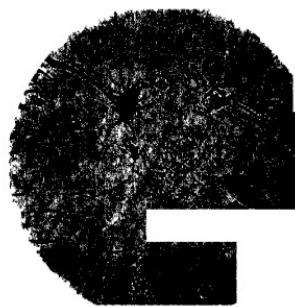
FIG. 5

FIGS. 2 TO 5. Photomicrographs of *dl*-glutamic acid. Figs. 2, 3, and 4 × 69.75; Fig. 5 × 16.5.

difficult to separate from the amino acid, was formed. If the amino group is protected by the phthalimido radical, as used by Sörensen and Andersen (16) for the synthesis of aminoadipic acid and by Dunn and Smart (17) for aspartic acid, hydrolytic cleavage of the group is so difficult that decomposition products are formed in addition to the desired amino acid.

As described in the experimental part, 8 gm. (51.8 per cent of the theoretical amount) of dl-glutamic acid (VII) of high purity were prepared. Photomicrographs of this product and of d-glutamic acid, isolated from ajinomoto, are shown in Figs. 1 to 5. On account of their low solubility in cold water both forms usually crystallize in balls or in needles, as shown by Keenan (18). However, when crystallization proceeds slowly, they form characteristic four-sided plates, needles, and pyramids as shown in Figs. 1 to 5 and as described by Fischer, Kropp, and Stahlschmidt (1).

Experiments for the preparation of glutamic acid through the reaction of sodium phthalimidomalonic ester with ethylene chlorohydrin were unsuccessful. Here, as found previously (17) for similar halogen compounds, the desired addition product could not be isolated although the liberation of chloride ions was complete, as shown by Volhard analyses. Similarly, all attempts to prepare pure sodium derivatives of either aminomalonic ester or benzoylaminomalonic ester were unsatisfactory. Such salts are easily formed from sodium and these esters in absolute ethyl alcohol or anhydrous toluene, but on exposure to air they are converted to impure, colored solids which react slowly and incompletely with β-chloropropionic ester.

<div style="text-align:center">EXPERIMENTAL</div>

Isonitrosomalonic Ester (II)—In making numerous preparations of isonitrosomalonic ester a high yield of crude product was easily obtained but the maximum yield of vacuum-distilled material was about 70 per cent of the theoretical. It was found necessary to distil the ester at pressures lower than 12 mm. to avoid decomposition. The most satisfactory yields of ester resulted when the sodium nitrite solution was allowed to drop slowly, over a period of about 3 days, into the malonic ester-acetic acid mixture contained in a long narrow tube. When the time of addition was extended to 9 days, the yield was not improved.

The vacuum-distilled product was a colorless oil, b.p. 150–152° at 4.8 mm.   Cerchez found 172° at 12 mm.

Aminomalonic Ester (III)—Isonitrosomalonic ester, in 100 gm. portions, was reduced with aluminum amalgam by the method of Cerchez.   The yield of vacuum-distilled product, b.p. 85–88° at 4 to 5 mm., was about 50 per cent.   On account of the instability of this almost colorless oil Cerchez converted it to the hydrochloride or the oxalate, but according to our experience the pure ester undergoes no apparent decomposition when preserved for many days in a glass-stoppered bottle.

Analysis
Calculated for $C_{17}H_{13}O_4N$.   N 8.00.   Found (Kjeldahl).   N 7.73, 7.73.

The reaction of aminomalonic ester with nitrous acid (Van Slyke method) was found to be about 60 per cent complete in 5 minutes and approximately 88 per cent in 4 hours.

The aluminum foil was amalgamated and the activity of the amalgam tested by methods devised by Dr. H. A. Spoehr and Dr. W. G. Young at the Division of Plant Biology, Carnegie Institution of Washington, Stanford University, California. Aluminum to be amalgamated is washed with warm, dilute sodium hydroxide solution until a vigorous evolution of gas occurs.   It is then washed with several portions of distilled water and once with 95 per cent ethyl alcohol.   A 1 per cent solution of mercuric chloride in 95 per cent ethyl alcohol is poured on the aluminum and left in contact with it for 20 seconds.   After the bichloride solution is decanted and the amalgam washed once with 95 per cent ethyl alcohol, the amalgam is ready for immediate use.

The activity of the aluminum is determined as follows: 2 gm. of aluminum pieces, amalgamated as described, are placed in a beaker and covered with 50 cc. of distilled water.   If the sample is active, a reaction begins at once, giving considerable heat and a strong evolution of hydrogen, and continues steadily until at the end of not more than 24 hours it is complete.   At this time all of the metal will have disappeared to give a fine precipitate of aluminum hydroxide.   Baker's aluminum foil, No. 11,530, was found to be more active than other samples of aluminum sheet or wire tested in this work or in the many experiments of Spoehr and Young.

Benzoylaminomalonic Ester (IV)—Aminomalonic ester was benzoylated in the usual manner. From 30 gm. of vacuum-distilled ester, a 47 gm. (98 per cent) yield of white, crystalline product, m.p. 62–63°, was obtained.

Analysis
Calculated for $C_{14}H_{17}O_5N$. N 5.02. Found (Kjeldahl). N 5.04, 4.94, 4.94, 4.96.

Propane-α-Benzoylamino-α,α,γ-Tricarboxylic Acid Triethyl Ester (V)—Purified sodium, 4.03 gm. (0.174 mol), was dissolved in 500 cc. of absolute ethyl alcohol and then 47 gm. (0.168 mol) of benzoylaminomalonic ester and 30.4 gm. (0.158 mol) of vacuum-distilled ethyl-β-bromopropionate (prepared by the method of Kendall and McKenzie (19)) were added to the cold alcoholic solution. The mixture, which became warm at once and deposited sodium bromide, was refluxed for 1½ hours on a water bath. According to Volhard analyses for halogen ions, approximately 45 minutes are required for the completion of the reaction. The alcohol was distilled and 100 cc. of water added to dissolve the sodium bromide. The lower oily layer was separated from the aqueous portion and the volatile material removed by distillation at 100° at 4 mm. The ester could not be distilled at 4 mm. without decomposition. The yield was 57.5 gm. (90 per cent).

Analysis
Calculated for $C_{19}H_{25}O_7N$. N 3.69. Found (Kjeldahl). N 3.77, 3.76, 3.74, 3.69.

Propane-α-Amino-α,α,γ-Tricarboxylic Acid Triethyl Ester (V-A)—β-Chloropropionic acid was prepared by the method of Jacobs and Heidelberger (20) and the acid esterified by the method used by Conrad (21) for chloroacetic ester. The triethyl ester derivative (V-A) was prepared essentially by the procedure described above for the triethyl ester (V). The yield of residual oil, dried for 2 hours at 110°, was 30 gm. (66 per cent), 28.7 gm. (0.164 mol) of vacuum-distilled aminomalonic ester being used. The product, which partly crystallized on standing, contained some yellow oil and was probably a mixture of the desired substance with some *ethane-α-carboxylic acid ethyl ester-β-iminomalonic ester (V-B)* and aminomalonic ester. However no evidence could

be obtained that the latter was present since an attempt to isolate glycine, as its benzoyl derivative, from the final acid hydrolysate was unsuccessful.

Analysis
Calculated for $C_{12}H_{21}O_6N$. N 5.09. Found (Kjeldahl). N 5.95, 5.88.

Glutamic Acid (VII). Method 1—The intermediate benzoyl-amino ester (V) and an excess of concentrated hydrochloric acid were refluxed in an all-glass apparatus for 16 hours, after which the elimination of carbon dioxide was complete, as shown by testing the evolved gas with barium hydroxide solution. After the solution was cooled and the precipitated benzoic acid filtered, the filtrate was heated to remove volatile material. The chloride ion content of the residual liquid was measured by a Volhard analysis of an aliquot portion and the chloride ions removed from the liquid with silver oxide. The filtrate from the silver chloride precipitate was evaporated to 100 cc., 200 cc. of ethyl alcohol were added, and the white, flocculent precipitate of *dl*-glutamic acid separated after cooling in an ice bath. The yield from 48.9 gm. of the intermediate ester was 8.01 gm., first crop, and 1.83 gm., second crop, or a total of 9.84 gm. (51.8 per cent). The melting point of the product, crystallized once from distilled water and dried at 110°, was 193.5–194.5° (corrected). When *dl*-glutamic acid is recrystallized many times, it melts at 198° (2) or 199° (1).

It is believed that the yield can be increased appreciably when larger quantities are used, as it was found by amino nitrogen analyses that about 1 gm. of glutamic acid was retained by the benzoic acid and silver chloride residues. Also about 10 per cent of the final glutamic acid solution was lost accidentally.

Analysis
Calculated for $C_5H_9O_4N$. N 9.52. Found (Van Slyke). N 9.64, 9.55.

Method 2—By a hydrolysis procedure identical with that described above 32 gm. of the intermediate substance (V-A) were refluxed with 50 cc. of concentrated hydrochloric acid for $14\frac{1}{2}$ hours. After further treatment the hydrolysate yielded a total of 5.9 gm. (36 per cent) of glutamic acid. This product was crystallized once from water and alcohol and dried at 110° for 15 hours.

Analysis

Calculated for $C_5H_9O_4N$. N 9.52. Found N (Kjeldahl) 9.42, 9.45; (Van Slyke) 9.56, 9.57.

The authors are indebted to Dr. W. G. Young of this department for valuable suggestions and for the loan of his catalytic hydrogenation apparatus.

SUMMARY

1. A new synthesis of glutamic acid has been described in which a 51.8 per cent yield of this amino acid has been obtained. Photomicrographs are given for the active and racemic modifications of glutamic acid, and their characteristic crystalline forms shown.

2. It has been found that the benzoyl radical effectively protects the amino group of aminomalonic ester when the latter is used for the synthesis of an aminodicarboxylic acid.

3. Hydrogen sulfide in alkaline solution proved to be an unsatisfactory agent for the reduction of isonitrosomalonic ester because the reduced product undergoes hydrolysis with the formation of aminomalonic acid.

4. Aminomalonic ester has been prepared in satisfactory yield from isonitrosomalonic ester and amalgamated active aluminum. Procedures have been devised for testing the activity of aluminum and for the preparation of aluminum amalgam. It is believed that the reducing activity of aluminum depends upon its state of purity rather than on any purely physical factor as claimed by Cerchez.

BIBLIOGRAPHY

1. Fischer, E., Kropp, W., and Stahlschmidt, A., *Ann. Chem.*, **365**, 181 (1909).
2. Wolff, L., *Ann. Chem.*, **260**, 79 (1890).
3. Knoop, F., and Oesterlin, H., *Z. physiol. Chem.*, **148**, 294 (1925).
4. Keimatsu, S., and Sugasawa, S., *J. Pharm. Soc. Japan*, No. 531, 34, 369 (1925).
5. Cerchez, V., *Bull. Soc. chim.*, series 4, **47**, 1279 (1930).
6. Johnson, T. B., and Nicolet, B. H., *J. Am. Chem. Soc.*, **36**, 345 (1914).
7. Baeyer, A., *Ann. Chem.*, **131**, 291 (1864).
8. Cerchez, V., *Bull. Soc. chim.*, series 4, **47**, 1282 (1930).
9. Dunn, M. S., and Schmidt, C. L. A., *J. Biol. Chem.*, **53**, 401 (1922).
10. Putochin, N. J., *Ber. chem. Ges.*, **56**, 2213 (1923).
11. Locquin, R., and Cerchez, V., *Bull. Soc. chim.*, series 4, **47**, 1274 (1930).

12. Adams, R., Voorhees, V., and Shriner, R. L., in Adams, R., Organic syntheses, New York, **8**, 92 (1928).
13. Adams, R., and Voorhees, V., in Adams, R., Organic syntheses, New York, **8**, 13 (1928).
14. Piloty, O., and Neresheimer, J., *Ber. chem. Ges.*, **39**, 514 (1906).
15. Keimatsu, S., and Kato, C., *J. Pharm. Soc. Japan*, **49**, 111, 123 (1929).
16. Sörensen, S. P. L., and Andersen, A. C., *Z. physiol. Chem.*, **56**, 236 (1908).
17. Dunn, M. S., and Smart, B. W., *J. Biol. Chem.*, **89**, 41 (1930).
18. Keenan, G. L., *J. Biol. Chem.*, **62**, 163 (1924–25).
19. Kendall, E. C., and McKenzie, B., in Clarke, H. T., Organic syntheses, New York, **3**, 51 (1923).
20. Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, **39**, 1465 (1917).
21. Conrad, M., *Ann. Chem.*, **188**, 217 (1877).

A CRYSTALLINE DERIVATIVE OF AN ACID PRESENT IN LIVER

A CORRECTION

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In this *Journal* an acid was described by West and Howe[1] which was believed to be a constituent of liver, active in causing a reticulocyte response in pernicious anemia. The acid was of peptide or diketopiperazine nature and on hydrolysis gave β-hydroxyglutamic acid, and γ-hydroxyproline was subsequently identified. It was isolated as a crystalline quinine salt which was believed to be clinically potent after repeated crystallization, although it was stated that, "Naturally the possibility of some adsorbed trace of highly active hormone cannot be entirely excluded at present."

The investigation of this substance has been beset with many difficulties, not only on account of technical problems in the way of preparing rigidly purified products, but also on account of the difficulty of obtaining an adequate number of suitable cases for testing the material clinically. We have, however, recently convinced ourselves that the original statements as to the clinical potency of the substance are erroneous and desire to withdraw them without delay. In addition to a number of positive clinical results we have obtained others which were completely negative in cases which subsequently responded in typical fashion to whole liver therapy. It seems reasonable to attribute more importance to these negative results than to the positive ones. In addition, it would appear that the statement that the "active material is not extracted" on shaking liver extract saturated with picric acid with butyl alcohol and ether must be modified, for we now find that a significant proportion of the active material is removed by this treatment and may be recovered by shaking the butyl alcohol-ether extract with dilute mineral acid.

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[1] West, R., and Howe, M., *J. Biol. Chem.*, **88**, 427 (1930).

STUDIES ON CRYSTALLIZED EGG ALBUMIN

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CONTENTS

INTRODUCTION

There is probably no protein whose physical-chemical properties have been more thoroughly studied than crystallized egg albumin, yet there have been surprisingly few amino acid determinations made on this protein since the early work of Osborne, Jones, and Leavenworth (1) and Abderhalden and Pregl (2). The exact determination of any of the amino acids in ovalbumin would be of great value in the calculation of the molecular weight of this protein.

The recent investigations of Parsons (3) and of others (4, 5) concerning the nutritive value of egg white and the nutritional disturbances resulting when it is fed have further indicated the desirability of a more complete knowledge of the amount of the individual amino acids in the various proteins present in egg

white. From this laboratory several investigations have been reported relating to different phases of embryonic metabolism. However, before the study of the changes occurring during embryonic development could be thoroughly and completely evaluated, it was necessary to know more about the chemical composition of the egg before embryonic development begins.

The present paper contains some analyses of a highly purified crystallized egg albumin. All the calculations of percentages in this investigation are made on the basis of an ash- and moisture-free protein.

TABLE I

Analyses of Crystallized Egg Albumin

	Maximum	Minimum	Average
	per cent	*per cent*	*per cent*
Phosphorus........................	0.099	0.096	0.097
Nitrogen..........................	15.23	15.03	15.12
Sulfur............................	1.38	1.33	1.36
H₂O..............................	6.10	5.12	5.60
Ash...............................	0.23	0.11	0.17
Ammonia..........................	1.41	1.32	1.39
Acid melanin*.....................	0.38	0.33	0.34
Humin............................	1.01	0.90	0.92

* Melanin nitrogen is the nitrogen present in the black precipitate which is insoluble in acid and is usually filtered off after hydrolysis of a protein. Humin nitrogen is the nitrogen present in the precipitate formed by making the filtrate from the melanin alkaline. Some proteins have very little of these present and some investigators call both melanin, while others call both humin.

Preparation of Crystallized Egg Albumin

The crystallized egg albumin was prepared and recrystallized twice according to the method of Sörensen and Höyrup (6). The crystallized albumin obtained from twenty-four eggs was dissolved in about 500 cc. of water and poured into 5 liters of boiling distilled water with rapid stirring so that the coagulated albumin was finely divided. It was then poured on several large filters and washed until the washings were *entirely* free of sulfates. The substance was then allowed to drain thoroughly, transferred to a flask containing about 10 volumes of 95 per cent boiling alcohol for an hour, and filtered. This was repeated once with 5 volumes

of 95 per cent alcohol and once with 5 volumes of absolute alcohol. The material was filtered and then stirred with 3 volumes of a good grade of ether and again filtered. This process was repeated twice and the protein was finally dried in a vacuum desiccator over sulfuric acid. This gave a dry white product which could easily be reduced to a powder. The yield was about 1 gm. per egg. In this manner several hundred gm. of the albumin were prepared, analyses of which are given in Table I.

The Basic Amino Acids

(a) Isolation after Butyl Alcohol Extraction

In view of the excellent results obtained by Dakin (7–9) when he used butyl alcohol to separate the monoamino acids from the basic and dicarboxylic amino acids, it was decided to make a preliminary extraction of the hydrolyzed protein with butyl alcohol before determination of the bases. According to Dakin (7) this should not cause a loss of the basic amino acids.

A 100 gm. sample of crystallized egg albumin was hydrolyzed by boiling for 30 hours with 5 times its weight of 20 per cent hydrochloric acid. The hydrolysate was concentrated under reduced pressure on a steam bath to remove as much hydrochloric acid as possible. More water was added and the concentration repeated. The remainder of the chlorides was removed by the addition of sulfuric acid and silver oxide. The solution was at all times kept strongly acid to Congo red by the addition of sulfuric acid. When the chlorides were all precipitated, the silver chloride was filtered or centrifuged off and the precipitate washed several times by boiling with very dilute (0.1 per cent) sulfuric acid. Analysis by the Kjeldahl-Gunning method indicated that only a small amount remained after about five such treatments. The washings were concentrated and added to the original mother liquors. These were then heated nearly to boiling and the silver removed as sulfide. The silver sulfide was centrifuged off and washed several times by boiling it with water and the filtrate and washings quantitatively freed from sulfuric acid by the addition of barium hydroxide. The barium sulfate was washed thoroughly by boiling it several times with water.[1] The filtrate and washings

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[1] It is usually best to boil the precipitate with very dilute sulfuric acid, similarly to the procedure used for the silver chloride. The sulfuric acid

were concentrated under reduced pressure to about 500 cc., the hydrogen ion concentration being adjusted to pH 7, and extracted at room temperature with butyl alcohol under reduced pressure for about 100 hours. The temperature of the boiling butyl alcohol was never above 50°. After 100 hours the extraction was not complete.

The extraction was continued for a second 100 hours and material continued to separate in the distillation flask. This material was kept separate from that obtained during the first 100 hours of extraction and examined separately. Some data are recorded below concerning the amino acids found in this fraction. Amino acids were still being extracted at the end of 200 hours when the extraction was discontinued. A total of 65 gm. of material was removed by the extraction with butyl alcohol.

The water solution containing the basic and dicarboxylic amino acids was concentrated to a small volume to remove the butyl alcohol. The dicarboxylic acids were removed by a method that will be described later under the discussion of the dicarboxylic acids. The basic amino acids were then determined by the author's modification of the method of Vickery· and Leavenworth (10). The amounts of these amino acids found are recorded in Table II.

In order to avoid as much as possible losses of these amino acids, during this entire investigation the solutions from which they were precipitated have been reexamined as described by Vickery and Leavenworth (11) and the small amounts usually found have been added to the main portions.

A second 100 gm. sample of crystallized egg albumin was treated as previously described with the following exceptions. First, the humin was removed by making the solution alkaline with barium hydroxide and filtering before removal of the chlorides in acid solution. The barium was then removed by excess sulfuric acid and then the chlorides were removed. Secondly, the silver chloride after being washed five times was decomposed with hydrogen sulfide, the silver sulfide washed, and the filtrate and washings, which contained 178.36 mg. of nitrogen, concentrated, and

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can be removed quantitatively with barium hydroxide and this precipitate, which should be much smaller, thoroughly washed with boiling water, and all the filtrates and washings concentrated and added to the original solution.

added to the main fraction of amino acids. Thirdly, the extraction with butyl alcohol was continued only for 60 hours. There were 37 gm. of material (monoamino acids) extracted during this period.

TABLE II

Basic Amino Acids of Crystallized Egg Albumin

Extraction by butyl alcohol	Amount isolated	Protein calculated on ash- and moisture-free basis	Per cent of total N
	per cent		
Arginine			
After 200 hrs...................	4.11	4.36	9.28
" 60 "	4.58	4.87	10.35
From butyl alcohol extract......	0.26	0.27	
Not extracted with butyl alcohol*........................	1.78	5.04	10.61
Not extracted with butyl alcohol........................	4.71	5.01	10.63
Histidine			
After 200 hrs...................	1.20	1.26	2.28
" 60 "	1.70	1.81	3.24
From butyl alcohol extract......	0.14	0.15	
Not extracted with butyl alcohol*........................	0.81	2.43	4.35
Not extracted with butyl alcohol........................	2.31	2.46	4.39
Lysine			
After 200 hrs...................	5.88	6.24	7.91
" 60 "	5.99	6.36	8.06
From butyl alcohol extract......	0.32	0.34	
Not extracted with butyl alcohol*........................	2.34	6.60	8.36
Not extracted with butyl alcohol........................	6.05	6.44	8.14

* Only 38 gm. of protein were used in this experiment.

The water solution containing the basic and dicarboxylic amino acids was investigated for arginine, histidine, and lysine as previously described except that the dicarboxylic acids were not removed. The results of this experiment are recorded in Table II.

(b) Bases in the Butyl Alcohol Extract

The differences between the values obtained for these amino acids in the first and second hydrolyses of albumin suggested the possibility that some of the basic amino acids were being extracted with butyl alcohol. Consequently tests were made on the material extracted by butyl alcohol to determine whether or not this might be so.

The very specific Sakaguchi color reaction for arginine which has been thoroughly studied by Weber (12) was used to test the various extracted materials for arginine and was found strongly positive in all cases. Since color reactions are not always dependable, it was decided to attempt the isolation of these amino acids as characteristic salts from the material obtained from the first 100 gm. of egg albumin during the second 100 hours of extraction.

The material (5.2 gm.), which was insoluble in butyl alcohol, was treated in the usual manner for isolation of the bases. The results of this experiment are recorded in Table II.

It will be observed that this period of extraction is much longer than that recommended by Dakin but three things must be kept in mind. In the first place, Dakin was dealing with proteins whose hydrolysis products may act differently during extraction with butyl alcohol; in the second place, the amount of material extracted depends not so much upon the time as it does upon the rate at which the butyl alcohol is distilled and consequently the amount that passes through the water solution in a given length of time; thirdly, the hydrogen ion concentration, as previously noted by Dakin, is a very important factor.

It seems wise at this time to point out to those who may be using, in feeding experiments, the material obtained from protein hydrolysates by extraction with butyl alcohol on the assumption that they are using pure monoamino acids that they may also be including small amounts of the bases. It is quite evident from this investigation that small amounts of bases are being extracted at the same time.

(c) Further Isolation Experiments

After the above findings further experiments were carried out to determine the basic amino acids without first extracting with

butyl alcohol. 38 gm. of crystallized egg albumin were hydrolyzed with hydrochloric acid and the hydrolysate examined for the bases by the method used in previous investigations (10). The Van Slyke (13) method on the arginine fraction gave 11.15 per cent of the total nitrogen as arginine nitrogen or 5.23 per cent as arginine in the protein. 5 gm. of arginine flavianate were obtained, equivalent to 1.783 gm. of arginine, 5.04 per cent of the protein as arginine.

Determination of the histidine by the colorimetric method of Koessler and Hanke (14) on the histidine fraction gave a value which was equivalent to 3.2 per cent of the total protein as histidine. There was only a very small amount of arginine present as indicated by the Van Slyke method (13). Cystine as calculated from the total sulfur in this fraction was equivalent to 120 mg., which was so small no attempt was made to remove it. The Folin and Ciocalteu (15) method indicated the absence of all but minute traces of tyrosine. After precipitation with mercuric sulfate 2.43 per cent of the protein was isolated as histidine by precipitation with flavianic acid.

The lysine was isolated as the picrate after precipitation as the phosphotungstate in the usual manner. 5.8 gm. of lysine picrate were obtained, equivalent to 2.35 gm. of free lysine. This is equivalent to 6.60 per cent of the protein as lysine. Another determination was made on 100 gm. of albumin with very similar results. The arginine found in this determination was 5.01 per cent of the protein. 2.46 per cent of the protein as histidine was isolated. Likewise the lysine was very nearly the same, since 6.44 per cent of the protein was found as lysine.

Proline

The proline was isolated from the combined butyl alcohol extracts obtained from the two 100 gm. runs previously mentioned in the discussion of the determination of "The basic amino acids." The various butyl alcohol fractions were combined after filtering off the insoluble material and washing it with cold absolute alcohol. The filtrates and washings were concentrated under reduced pressure to remove the alcohol and the sticky brown residue boiled with about 10 to 20 parts of absolute ethyl alcohol. Not all of the material was soluble, so the insoluble material was added to the

monoamino acid fraction. The alcoholic solution was again evaporated on a steam bath under reduced pressure and again taken up in absolute alcohol. Practically the whole of the residue was soluble in the hot absolute alcohol. On standing overnight, about 1.5 gm. of material crystallized out. This was filtered off and washed with absolute alcohol; it will be examined later. The alcoholic filtrate was concentrated under reduced pressure to a thick syrup, taken up in boiling water, boiled with norit, filtered, and allowed to stand overnight. A small amount of material separated and was added to the material which crystallized from the alcohol.

From the clear aqueous solution which should contain all of the proline, 9.2 gm. of material were obtained by evaporation in a vacuum desiccator. A determination of the amino nitrogen present showed 15 per cent of the total nitrogen present as amino nitrogen. Since it has been pointed out by Dakin and others that all the non-amino nitrogen at this point may not be proline nitrogen, it was considered advisable to convert the proline to the picrate by the method of Town (16). This was done and 4.15 per cent of the protein as proline was isolated as the picrate.

Tyrosine, Tryptophane, and Cystine

A summary of the results obtained by other investigators for these amino acids is recorded in Table III and the method used is also indicated there. Averages of the results obtained in this investigation, which the author considers most accurate, are also recorded in Table III for comparison. The average value for cystine obtained by the method of Folin and Marenzi (17) was 1.33 per cent. The tyrosine and tryptophane were determined by the method of Folin and Ciocalteu (15). The value obtained for tyrosine was 4.21 per cent; that for tryptophane was 1.28 per cent.

In order to determine the tyrosine by isolation, 100 gm. of crystallized egg albumin were hydrolyzed with hydrochloric acid in the usual manner. The solution was concentrated to remove most of the hydrochloric acid, diluted, and filtered to remove the acid melanin. The filtrate was concentrated again, the residue taken up in water, and the chlorides removed with silver oxide and sulfuric acid. The silver chloride was washed several times

TABLE III

Summary of Determinations by Other Investigators of Some Amino Acids of Crystallized Egg Albumin and Average of Author's Most Accurate Determinations

Amino acid	Determination by other investigators			Author's determination	
	per cent	Bibliographic No.	Method	per cent	Method
Arginine*	4.91	1	Kossel and Patten	5.03	Isolation as flavianate
	6.00	19	Direct precipitation as flavianate		
	2.90	20	Kossel and Patten		
Histidine	1.71	1	" " "	2.44	Isolation as flavianate
	2.30	21	Colorimetric, Koessler and Hanke		
	1.50	20	Kossel and Patten		
Lysine	3.76	1	Isolation as picrate	6.41	Isolation as picrate
	3.90	20	" "		
Tyrosine	1.77	1	"	4.21	Folin and Ciocalteu
	1.10	2	"	3.20	Isolation
	0.99	20	"		
	4.20	22	Folin and Looney		
	4.00	15	" Ciocalteu		
	2.35	21	Koessler and Hanke		

Amino acid	Determination by other investigators			Author's determination	
	per cent	Bibliographic No.	Method	per cent	Method
Cystine	0.81	22	Colorimetric, Folin and Looney	1.83	Folin and Marenzi
	1.22	17	Folin and Marenzi		
	1.19	23	Okuda		
	1.07	23	Sullivan		
	0.20	1	Isolation		
	0.35	24	"		
	0.40	25	"		
Tryptophane	1.23	22	Folin and Looney	1.28	Folin and Ciocalteu
	1.33	15	" " Ciocalteu		
	Trace	1	Isolation		
	"	26	"		
Proline	3.56	1	By difference in amino and non-amino N	4.15	Isolation as picrate
	2.25	2	Isolation		
	1.50	20	"		
Glutamic	13.30	18	"	13.96	Isolation as hydrochloride
	8.50	27	"		
	8.00	2	"		
	9.10	1	"		
Aspartic	6.20	18	Isolation	6.07	Isolation as copper salt
	1.70	27	"		
	1.50	1	"		
	2.20		"		
Hydroxyglutamic	Absent	2	"	1.36	Isolated as free acid
Acid melanin	0.29	28	Insolubility in acid	0.34	Insolubility in acid
Humin				0.92	" " dilute alkali
Ammonia	1.34	28	Liberation with weak alkali at low temperature	1.39	Liberation with weak alkali at low temperature

* Hunter and Dauphinee (18) analyzed crystallized egg albumin by the very specific arginase method and found that the arginine nitrogen was equivalent to 10.36 per cent of the total nitrogen. This is higher than most results reported in the literature but Hunter and Dauphinee did not give the nitrogen content of the protein so there is no way to determine what the per cent of arginine was in the sample of protein used by them.

with boiling dilute (0.1 per cent) sulfuric acid and the washings added to the original solution. The pH of the solution was adjusted to faint reaction to Congo red by the addition of barium hydroxide. The barium sulfate was filtered off and washed thoroughly with boiling water. The filtrate and washings were concentrated under reduced pressure until amino acids began to crystallize out. This concentrated solution was allowed to stand in the ice box overnight, filtered, and further concentrated and again allowed to stand. This was repeated until Millon's test was negative on the filtrate. The precipitate, which was composed of tyrosine and large quantities of other amino acids, was dissolved in boiling water and boiled with norit. The tyrosine separated more readily from this solution. The first crop of crystals was filtered off and the filtrate concentrated and allowed to stand in the ice box. By repetition of this process several times 3.2 per cent of the weight of the protein was obtained as practically pure tyrosine. The nitrogen content was 7.67 per cent, while the theoretical per cent is 7.74.

Aspartic and Glutamic Acids

For the determination of these amino acids the early investigators used the ester method, but more recent investigators have found the method of Foreman (29) or some modification of it a great step in advance over the older methods. Dakin (8) modified the method of Foreman by substituting barium for calcium and this modification is the one generally used at the present time with only slight alterations. Dakin (8) found that preliminary precipitation of the bases with phosphotungstic acid materially decreased the yields of aspartic and glutamic acids, but Jones and Moeller (30) report the highest values ever obtained for these acids and their general procedure includes preliminary precipitation of the basic amino acids with phosphotungstic acid and removal of the phosphotungstic acid from the filtrate with amyl alcohol and ether. In general, their procedure has been followed in this investigation. The results of earlier investigators for these amino acids are summarized along with those of the author in Table III.

In this investigation the aspartic and glutamic acids have been determined under four different conditions. They were first

determined in the solution which had been extracted 200 hours with butyl alcohol under reduced pressure, as previously mentioned in the discussion of "The basic amino acids." The procedure of Jones and Moeller (30) was followed exactly except that the preliminary precipitation of the bases with phosphotungstic acid was not carried out.

The yields were very low in comparison to those obtained by Jones and Moeller. 7.36 gm. of glutamic acid were isolated as the hydrochloride, which is equivalent to 7.81 per cent of the weight of the protein as glutamic acid. 3.8 gm. of aspartic acid were isolated as the copper salt, which is equivalent to 4.03 per cent of the albumin as aspartic acid.

The second determination was made on 100 gm. of crystallized egg albumin which after hydrolysis had been extracted for 60 hours with butyl alcohol to remove most of the monoamino acids as previously mentioned under "The basic amino acids." In this experiment the arginine and histidine were removed as silver salts by the usual procedure. The lysine was then removed from the filtrate by phosphotungstic acid in the usual manner. The phosphotungstic acid was removed with amyl alcohol and ether, and from this point the procedure of Jones and Moeller (30) was followed exactly. The amount of glutamic acid hydrochloride obtained was 9.6 gm., which is equivalent to 7.68 gm. of glutamic acid or 8.15 per cent of the protein as glutamic acid. The aspartic acid was weighed as the copper salt and 8.7 gm. were obtained. This is equivalent to 3.56 gm. of aspartic acid or 3.78 per cent of the weight of the protein.

The two following experiments clearly demonstrate that the above yields of glutamic and aspartic acids are not nearly the maximum yields of these acids that can be obtained from crystallized egg albumin.

In the first of these experiments 100 gm. of crystallized egg albumin were used for the preparation of tyrosine, as previously described. The filtrates and washings from the tyrosine precipitates were all combined, concentrated under reduced pressure, and the bases removed with phosphotungstic acid. The exact procedure of Jones and Moeller (30) was then followed for the determination of aspartic and glutamic acids. The weight of glutamic acid obtained as hydrochloride was 12.45 gm., which is

equivalent to 13.22 per cent of the weight of the albumin as glutamic acid. 5.33 gm. of aspartic acid were obtained as the copper salt, which is equivalent to 5.66 per cent of the protein as aspartic acid.

In the second experiment 50 gm. of crystallized egg albumin were hydrolyzed with 20 per cent hydrochloric acid (300 cc.) for 36 hours. The hydrolysate was concentrated to a thick syrup under reduced pressure to remove most of the hydrochloric acid. The thick syrup was taken up in 400 cc. of water and filtered to remove the acid melanin. The melanin was washed thoroughly and the filtrate and washings concentrated to a thick syrup. The syrup was taken up in 300 cc. of water and the glutamic and aspartic acids were precipitated in the usual manner without previous removal of the basic amino acids with phosphotungstic acid. A total yield of 8.22 gm. of glutamic acid hydrochloride was obtained, which is equivalent to 6.58 gm. of glutamic acid or 13.96 per cent of the weight of the protein. The yield of copper aspartate was 6.97 gm. This is equivalent to 2.86 gm. of aspartic acid or 6.07 per cent of the protein.

From these experiments it appears quite conclusively that after a preliminary extraction with butyl alcohol not only may the maximum yield of these amino acids from protein fail to be obtained but rather that actual losses may result.[2]

Hydroxyglutamic Acid

The residues from the aspartic and glutamic acid determinations from 250 gm. of crystallized egg albumin were combined and an excess of sulfuric acid was added. The copper was removed as the sulfide and the filtrate and washings were concentrated under reduced pressure to remove hydrogen sulfide. The chlorides were then removed by the addition of silver oxide, care being taken that the solution was kept acid with sulfuric acid. The excess silver was removed from the filtrate and washings as the

[2] In their investigations of the basic amino acids, Vickery and Leavenworth (*J. Biol. Chem.*, 76, 707 (1928)) found that aspartic acid appeared in large quantities in the histidine fraction. This would explain the low results obtained for this amino acid in this investigation when the silver procedure for the bases was used before the aspartic acid was determined. It would not, however, explain the low results in the other experiments.

sulfide. The filtrate and washings from the silver sulfide were concentrated to about 100 cc. and made alkaline with barium hydroxide. The barium sulfate was filtered off and washed twice by boiling with 50 cc. of water. About 10 gm. of pure barium hydroxide were added to the filtrate and washings which were already slightly alkaline, and the whole solution poured into a liter of alcohol and allowed to stand in an ice box for 2 days. The precipitate was filtered off and washed once with alcohol. It was then dissolved in water and the barium quantitatively removed with sulfuric acid. The filtrate and washings from the barium sulfate were concentrated to a thin syrup under reduced pressure at a low temperature and then to dryness in a vacuum desiccator over sulfuric acid for several days. The dry white material obtained at this point was ground up with glacial acetic acid at a temperature of 30–35° for about an hour. This was repeated twice. The glacial acetic acid extract was poured into 10 volumes of absolute ethyl alcohol, and the precipitate which formed was centrifuged off and washed twice with absolute alcohol and twice with anhydrous ether and dried for 2 days in a vacuum desiccator over sulfuric acid. 3.2 gm. of a dry hygroscopic powder were obtained. Its solution was strongly acid to litmus.

When titrated, 0.0401 gm. required 1.86 cc. of 0.1317 N sodium hydroxide to neutralize it to phenolphthalein. From these data, on the assumption that one carboxyl group was titrated, the molecular weight of the compound was calculated to be 163.7. The molecular weight of hydroxyglutamic acid is 163.

The silver salt was then prepared by Dakin's method (7) and analyzed. 0.3652 gm. gave 0.2774 gm. of silver chloride, which is equivalent to 0.2087 gm. of silver or 57.16 per cent. The theoretical value is 57.28 per cent. The theoretical value for nitrogen is 3.72 per cent; the value found by the Kjeldahl-Gunning method was 3.66 per cent.

The strychnine salt was also made and analyzed. The theoretical value for nitrogen is 8.45 per cent; the value found was 8.38 per cent.

DISCUSSION

The values found in this investigation for the basic amino acids agree quite well with some of the best values in the literature but

in most cases the present values are higher. The arginine value is higher than all others except that of Fürth and Deutschberger (19) who reported 6 per cent. It is even higher than the figure obtained by the very specific arginase method of Hunter and Dauphinee (18). Their value, calculated on the basis of total nitrogen, was 10.36 per cent and the average of the two best determinations made in this investigation was 10.62 per cent.

The value for histidine is distinctly higher than any previously reported.

The lysine value is somewhat higher than those reported in the literature; however, since such large quantities of protein were used in each case and the melting point of the picrate, in which form the lysine was isolated and weighed, was 257° or above, it probably represents nearly the true value for lysine in egg albumin.

In a private communication from Dr. H. B. Vickery after the experimental work reported in this investigation was finished, I learned that an analysis of crystallized egg albumin for the basic amino acids was practically finished in his laboratory. The values from the two laboratories are in quite good agreement for arginine and lysine, his arginine values being higher and lysine values lower than those I have found. There is a marked difference in the histidine values, those from this laboratory being much higher than those from the New Haven laboratory. At Dr. Vickery's (31) suggestion that the flavianic acid might have been contaminated (a difficulty which he had encountered) I have reanalyzed the histidine flavianate and find that it is quite pure. Probably the only significant difference in the procedures in the two laboratories is the use of the copper procedure which Dr. Vickery employs for the removal of cystine from the histidine fraction and which was not used in this investigation. As stated in the experimental part, the histidine fraction in this investigation was analyzed for cystine by the Folin and Marenzi (17) method and also for total sulfur and only very small quantities were found.

The colorimetric values for tryptophane (1.28 per cent), tyrosine (4.21 per cent), and cystine (1.33 per cent) are in quite good agreement with those already reported in the literature and determined by the same methods. The isolation value for tyrosine (3.2 per cent) is much higher than any previous isolation value and it is quite possible that this value could be still further increased with an improved method.

The value for proline (4.15 per cent) is higher than those previously reported, probably due to the improvement in the methods for isolating this amino acid.

The glutamic acid and aspartic acid percentages (13.96 and 6.07 per cent respectively) are in quite good agreement with the 13.3 per cent for glutamic acid and the 6.20 per cent for aspartic acid obtained by Jones and Moeller (30). From the data reported in the experimental part it is quite obvious that the maximal yield of these amino acids cannot be obtained after the butyl alcohol extraction procedure has previously been carried out on a solution containing them.[3] At present it is not possible to state whether the bases should be removed before precipitation of the barium salts of the dicarboxylic amino acids.[2]

The discovery of the presence of hydroxyglutamic acid is not surprising since it has been found in several proteins and seems to be a general protein constituent. It was not found in large quantities but was unmistakably present. It is probably there in greater amount than the 1.36 per cent reported in this paper, for reasons already mentioned in the experimental part.

A summary of the results of this investigation as well as a number of values found by previous investigators for each of the amino acids is recorded in Table III.

Calculation of Molecular Weights and Percentage of Amino Acids in Proteins

The amounts of certain of the amino acids in the protein molecule have been used by recent investigators as a basis for the calculations of molecular weights. The validity of such calculations depends entirely upon two considerations. In the first place, the method for the determination of the amino acid in question must be accurate, and in the second place, the number of mols of the amino acid present in the protein molecule must not be too large.

[3] The abstract of a paper presented by R. M. Hill before the Biological Division of the American Chemical Society at Buffalo, August 30 to September 4, states that a solution of glutamic acid was extracted from 18 to 36 hours and that from 3 to 36 per cent of the glutamic acid was extracted by butyl alcohol. This is exactly in accord with the results reported in this paper on the amounts of glutamic and aspartic acids found when the protein hydrolysate was not extracted with butyl alcohol and after a similar hydrolysate had been extracted with butyl alcohol.

The second of these conditions depends entirely upon whether or not the amino acid is present in large quantities in the particular protein under investigation since it is well known that the amounts of the various amino acids vary with the individual proteins.   If the amino acid is present in a greater amount than 2 to 3 per cent, there will usually be too many mols present to be of much value in the calculation of molecular weights.   This is well illustrated in Table IV if we take, for example, the amino acid histidine and assume that the method of isolation used in this investigation has given the correct value, which was found to be 2.44 per cent. Under these circumstances, if the molecular weight of egg albumin is 34,000, it is impossible to say whether there are 5 or 6 mols present since 5 mols would be equivalent to 2.28 per cent and 6 mols would be equivalent to 2.73 per cent.   The same is true for the aspartic acid.   Either 15 or 16 mols would probably fall within the range of experimental error for the best methods for determination of molecular weights.

In the discussion of the calculations of the molecular weights of proteins from the percentages of the amino acids present there is another fact which should be emphasized, the fact that the method of calculation of the percentages of the amino acids in the protein molecule is incorrect and that the values usually reported are not the actual values present.

It is quite generally considered that the peptide linkage is the most important linkage in the protein molecule and also granted whatever the linkage that on hydrolysis water is added.   If this is true, each amino acid on hydrolysis of the protein must gain in weight by 18, the molecular weight of water.   This must be true for all the amino acids except those at the end of the chain if the protein molecule has a straight chain structure.   Of these the one with the free amino group should gain 17 (an OH) and the one with the free carboxyl group should gain 1 (an H).   It is useless to speculate concerning these amino acids until we know more about which ones are likely to be on the end of the chain, for, if we assume that the minimal molecular weight of egg albumin is about 34,000, this can only mean two amino acids out of approximately 250. In that case the chances are only 1 in 125, for example, that it would be tryptophane at the end of the chain since there are probably only 2 mols of this amino acid in 1 molecule of egg albumin.

If it is considered that each amino acid adds a molecule of water during hydrolysis of the protein and that there are approximately

TABLE IV

Number of Mols and Percentages of Some Amino Acids in Crystallized Egg Albumin

Amino acid (1)	Average determinations (2)	Corrected for water (3)	No. of amino acid mols estimated (4)	Corrected for water (5)	Not corrected for water (6)
	per cent	per cent		per cent	per cent
Tryptophane	1.28	1.17	3	1.65	1.81
			2	1.10	1.21
Tyrosine	4.21	3.79	9	4.31	4.79
			8	3.83	4.25
Cystine	1.33	1.22	2	1.20	1.41
			3	1.66	2.11
			9	4.10	4.59
Arginine	5.03	4.51	10	4.58	5.12
			11	5.10	5.62
Lysine	6.41	5.63	14	5.42	6.16
			15	5.80	6.58
			16	6.23	7.02
Histidine	2.44	2.15	5	2.00	2.28
			6	2.42	2.73
Glutamic	13.96	12.25	32	12.15	13.98
			33	12.52	14.26
Aspartic	6.07	5.25	15	5.10	5.86
			16	5.41	6.26
Proline	4.15	3.49	12	3.35	3.99
			13	3.63	4.32
Hydroxyglutamic	1.36	1.21	2	0.85	0.93
			3	1.26	1.44
			4	1.78	1.92

In Column 2 are listed the percentages found in the present investigation. The percentages in Column 3 are calculated from the values in Column 2 by correction for the addition of 1 mol of water per mol of amino acid during hydrolysis. The values in Column 5 are the percentages, corrected for water, and in Column 6 those uncorrected for water, which correspond to the number of mols listed in Column 4 and calculated on the basis of 34,000 as the molecular weight of crystallized egg albumin.

250 mols of amino acids present in 1 molecule of egg albumin, roughly, then, the weight of the hydrolysis products of 1 gm. mole-

cule of egg albumin is not 34,000 gm. but 38,500 gm., which might make a marked difference in the calculation of the number of mols of certain of the amino acids present. For example, if the true percentage of glutamic acid present was 13.96, then instead of 31 or 32 mols fitting the theory best it would require 36 or 37 mols, and instead of 2 mols of tryptophane being equal to 1.21 per cent they would only be equal to 1.10 per cent of the entire weight of the molecule of albumin, while 3 mols would be 1.65 per cent instead of 1.81 per cent.

However, there is usually no error made in the calculations of molecular weights for two reasons. First, because the percentages of the amino acids, being incorrectly calculated, are used along with the weights of the amino acids after hydrolysis (true molecular weight) and not the weight that is present in the protein molecule. Consequently there is no error in the molecular weight determined, or rather there is compensation of errors. For example, if the per cent of cystine is 1.33 and it is assumed that there are 2 mols per molecule of egg albumin, then the minimal molecular weight is approximately 36,000 if the cystine is not corrected for water added during hydrolysis. If the cystine is corrected for water, then the per cent present is 1.22 and the molecular weight would not be calculated on the basis of 2 mols (480) but on the basis of 2 mols less 36 (444) and the minimal molecular weight still would be approximately 36,000. In the second place, the number of mols is usually chosen which best fits the theory and this is quite simple for those amino acids which are present in small amounts.

The greatest error comes, as already pointed out, in the failure to calculate the actual percentages of the amino acids present in the protein molecule. Figures are given in Table IV to illustrate some of the points which have just been discussed.

SUMMARY

1. A very pure sample of crystallized egg albumin has been analyzed for ash, sulfur, and nitrogen and the results are lower in general than those previously reported by other investigators. This may be due to a more complete removal of ammonium sulfate.

2. Arginine and histidine were determined by isolation as flavianates, while lysine was determined as the picrate. The values

for arginine and lysine are slightly higher than most of the values found in the literature, while the histidine value is much higher.

3. The butyl alcohol extract was examined for the basic amino acids and all three were isolated and definitely identified as characteristic derivatives, showing that they may be removed by prolonged extraction with butyl alcohol.

4. Proline was isolated in larger quantities than have previously been reported, 4.15 per cent being obtained.

5. Tyrosine, tryptophane, and cystine were determined colorimetrically and the values agree very closely with those previously reported in the literature. The sulfur of the cystine as determined colorimetrically is only equivalent to 26.06 per cent of the sulfur present in the albumin.

6. An attempt was made to isolate tyrosine quantitatively and 3.2 per cent was isolated, a much higher value than any previously reported.

7. The glutamic and aspartic acids were isolated as hydrochloride and copper salt respectively, before and after butyl alcohol extraction of the hydrolysate. The values obtained before butyl alcohol extraction are higher than those obtained afterward and agreed quite closely with those of Jones and Moeller.

8. Hydroxyglutamic acid was isolated, 1.36 per cent being obtained. This is the first time this amino acid has been obtained from egg albumin.

9. The analytical results are briefly discussed and the values obtained are compared with those obtained by other investigators.

10. Some molecular weight calculations and considerations are made and discussed.

BIBLIOGRAPHY

1. Osborne, T. B., Jones, D. B., and Leavenworth, C. S., *Am. J. Physiol.*, **24**, 252 (1909).
2. Abderhalden, E., and Pregl, F., *Z. physiol. Chem.*, **46**, 24 (1905).
3. Parsons, H. T., *J. Biol. Chem.*, **90**, 351 (1931).
4. Boas, M. A., *Biochem. J.*, **18**, 422, 1321 (1924); **21**, 712 (1927).
5. Fixsen, M. A. B., *Biochem. J.*, **25**, 596 (1931).
6. Sörensen, S. P. L., and Höyrup, M., *Compt. rend. trav. Lab. Carlsberg*, **12**, 12 (1917).
7. Dakin, H. D., *Biochem. J.*, **12**, 290 (1918).
8. Dakin, H. D., *J. Biol. Chem.*, **44**, 499 (1920).
9. Dakin, H. D., *Z. physiol. Chem.*, **130**, 159 (1923).

10. Calvery, H. O., *J. Biol. Chem.*, **83**, 631 (1929).
11. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **79**, 377 (1928).
12. Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930).
13. Van Slyke, D. D., *J. Biol. Chem.*, **10**, 15 (1911).
14. Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, **39**, 497, 521, 539 (1919).
15. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
16. Town, B. W., *Biochem. J.*, **22**, 1083 (1928).
17. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
18. Hunter, A., and Dauphinee, J. A., *J. Biol. Chem.*, **85**, 627 (1929–30).
19. Fürth, O., and Deutschberger, O., *Biochem. Z.*, **186**, 139 (1927).
20. Skraup, Z. H., and Hummelberger, F., *Monatsh. Chem.*, **30**, 125 (1909).
21. Hanke, M. T., *J. Biol. Chem.*, **66**, 489 (1925).
22. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
23. Sullivan, M. X., and Hess, W. C., *Pub. Health Rep., U. S. P. H. S.*, suppl. 86 (1930).
24. Harris, L. J., *Proc. Roy. Soc. London, Series B*, **94**, 441 (1923).
25. Mörner, K. A. H., *Z. physiol. Chem.*, **34**, 207 (1901–02).
26. Hopkins, F. G., and Cole, S. W., *J. Physiol.*, **27**, 418 (1901–02).
27. Hugounenq, L., and Morel, A., *Compt. rend. Acad.*, **142**, 1426 (1906); *Bull. Soc. chim.*, series 4, 1, 154 (1907).
28. Osborne, T. B., and Harris, I. F., *J. Am. Chem. Soc.*, **25**, 323 (1903).
29. Foreman, F. W., *Biochem. J.*, **8**, 463 (1914).
30. Jones, D. B., and Moeller, O., *J. Biol. Chem.*, **79**, 429 (1928).
31. Vickery, H. B., private communication.

VITELLIN OF HEN'S EGG

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Vitellin, the chief protein of egg yolk, has for a long time been of great interest to biologists because of its close relationship to the developing avian embryo. For this and other reasons it has also been of interest to chemists. It has been analyzed by four sets of investigators with very different results. The values obtained by Levene and Alsberg (1) and by Hugounenq (2) do not agree with those of Abderhalden and Hunter (3) and Osborne and Jones (4). The values obtained by Osborne and Jones are probably the most accurate since they are the most recent.

Because of our interest in the chemical changes that occur during embryonic development, and since we have large quantities of vitellin available, we decided to investigate its amino acid content by some of the more modern methods. We also decided to determine the distribution of the nitrogen by the method of Van Slyke (5) since it had never been reported, although Plimmer and Rosedale (6) made such a study on the combined egg yolk proteins.

EXPERIMENTAL

Preparation of Vitellin

The vitellin was prepared essentially according to the method of Osborne and Jones (4). The yolks of twenty-four eggs were separated from the whites and washed thoroughly with saline solution and running water without rupture of the yolk membrane. They were then broken into an equal volume of 10 per cent sodium chloride solution and extracted with ether containing about 2 per cent ethyl alcohol until no more lipids could be obtained in the ether layer. The water solution was then strained through cheesecloth to remove the yolk membranes which are insoluble in sodium chloride solutions. After removal of the yolk membranes the

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solution was poured into 20 volumes of water and allowed to stand overnight. The supernatant liquid was siphoned off and the remaining precipitate and fluid centrifuged. Without washing, the precipitate was dissolved in 10 per cent sodium chloride and again precipitated by pouring into 20 volumes of water. The suspension was allowed to stand overnight and the supernatant fluid removed by means of a siphon. The remaining material was centrifuged and the precipitate washed once with water. It was then suspended in 5 liters of 80 per cent alcohol, heated to

TABLE I

Ash, Moisture, Nitrogen, Phosphorus, Sulfur, and Some Amino Acids of Vitellin

Substance	Author's values by special methods	Osborne and Jones	Hugou-nenq	Levene and Alsberg
	per cent	per cent	per cent	per cent
Ash..........................	0.32	3.19		
Moisture......................	5.86	10.50		
Nitrogen......................	15.03	15.50		
Phosphorus...................	0.92	0.94		
Sulfur........................	0.95	1.02		
Tyrosine......................	5.01	3.37	2.00	0.40
Tryptophane..................	1.24			
Cystine.......................	1.19 (1.59)*			
Arginine......................	7.77 (8.05)*	7.46	1.00	1.20
Histidine.....................	1.22 (0.92)*	1.90	2.10	Trace
Lysine........................	5.38 (8.73)*	4.81	1.20	2.40

* These values were calculated from the per cent of nitrogen obtained in the Van Slyke distribution study recorded in Table II.

boiling, and kept nearly at the boiling point by means of a good steam bath for several hours. The precipitate was filtered as dry as possible with suction and the process repeated twice, once with 95 per cent alcohol and once with absolute alcohol. After filtering the precipitate as dry as possible on a Buchner funnel, it was washed several times with ether and finally dried in a vacuum desiccator over sulfuric acid for several days. The yield was 35 to 40 gm. from twenty-four eggs. In this manner several hundred gm. of vitellin were prepared which had the ash, moisture, phosphorus, nitrogen, and sulfur content shown in Table I.

Distribution of Nitrogen

The study of the distribution of nitrogen was made by the method of Van Slyke (5). It was modified for the determination of arginine according to the suggestion of Plimmer and Rosedale (7) and the modified apparatus suggested by Koehler (8) was used. The results of three experiments are given in Table II. Since this is the first time the nitrogen distribution has been studied, the values cannot be compared with others. However, from the percentage of nitrogen obtained by this method the percentages of the amino acids in the protein were calculated and compared (Table I) with those obtained by other methods in this investigation.

TABLE II

Distribution of Nitrogen in Vitellin Determined by Van Slyke Method

The values are expressed in per cent of the total nitrogen.

Experiment No.............................	I	II	III
	per cent	*per cent*	*per cent*
Acid melanin N...................	0.51	0.47	0.47
Amide N........................	9.19	9.25	9.21
Humin "	1.45	1.02	1.23
Arginine N	16.58	16.64	16.53
Histidine "	1.76	1.76	1.50
Lysine N	11.04	11.30 ·	11.11
Cystine "	1.23	1.26	1.24
Amino N filtrate..................	55.31	55.50	55.00
Non-amino N filtrate..............	3.34	2.62	3.08
Total regained..................	100.41	99.82	99.37

Individual Amino Acids

All values for the amino acids reported in this investigation are calculated on an ash- and moisture-free basis. Before analysis the vitellin was ground to a powder and passed through a 40 mesh sieve. It was analyzed for cystine by the method of Folin and Marenzi (9) and for tyrosine and tryptophane by the method of Folin and Ciocalteu (10). An average of the values obtained is reported in Table I.

Two samples were used for the estimation of the basic amino acids by a method previously described (11). In the first analysis

of a sample equivalent to 36.9 gm. of ash- and moisture-free vitellin the arginine flavianate obtained was equivalent to 7.64 per cent of the protein as arginine. The nitrogen content of the flavianate was 17.36 per cent; theoretical, 17.21 per cent. The histidine was lost before the flavianate was obtained. The lysine picrate had a melting point of 262° and was equivalent to 5.36 per cent of the protein as lysine.

The second sample of vitellin used was equivalent to 100.1 gm. of ash- and moisture-free protein. The arginine flavianate obtained in this run was equivalent to 7.91 per cent of the protein as arginine. The nitrogen content was 17.31 per cent, while the theory requires 17.21 per cent. The amount of histidine flavianate obtained was equivalent to 1.22 per cent of the protein as histidine. The nitrogen content of the histidine flavianate was 12.54 per cent, while 12.52 per cent is required by theory. The amount of lysine picrate obtained was equivalent to 5.43 per cent of the protein as lysine. The melting point of the picrate was 260°, while the melting point of pure lysine picrate is 267°. An average of the values obtained for the basic amino acids is recorded in Table II.

<div align="center">DISCUSSION</div>

It is interesting to note that although our method of preparation of vitellin was very similar to that of Osborne and Jones, there is a marked difference in the amounts of moisture and ash present and also a distinct difference in the nitrogen content of the two preparations. This is not true of the sulfur and phosphorus, however, for these values are easily within experimental error for duplicate determinations, even in the same laboratory.

Our value for tyrosine is much higher than any of the other values recorded in Table I, undoubtedly due to the fact that it was obtained by a colorimetric method and the others are values obtained by isolation methods. It agrees closely with the value (5.02 per cent) obtained by Folin and Denis (12) in their early investigations of the tyrosine content of various proteins by a colorimetric method.

The values for arginine, histidine, and lysine are not at all in agreement with the values of Hugounenq (2) and Levene and Alsberg (1), which are undoubtedly incorrect. In consideration of

the fact that Osborne and Jones (4) used the Kossel and Kutscher (13) procedure, there is good agreement between their values and ours.

SUMMARY

1. The preparation of vitellin is described and a sample was analyzed for ash, moisture, nitrogen, phosphorus, and sulfur.

2. The nitrogen distribution in vitellin was determined by the method of Van Slyke.

3. The tyrosine, tryptophane, and cystine contents of vitellin were determined by colorimetric methods.

4. Arginine, histidine, and lysine were determined by isolation as well characterized crystalline derivatives and the results contrasted with those of previous investigators.

BIBLIOGRAPHY

1. Levene, P. A., and Alsberg, C. L., *J. Biol. Chem.*, **2**, 127 (1906–07).
2. Hugounenq, L., *Compt. rend. Acad.*, **142**, 173 (1906).
3. Abderhalden, E., and Hunter, A., *Z. physiol. Chem.*, **48**, 505 (1906).
4. Osborne, T. B., and Jones, D. B., *Am. J. Physiol.*, **24**, 153 (1909).
5. Van Slyke, D. D., *J. Biol. Chem.*, **10**, 15 (1911–12).
6. Plimmer, R. H. A., and Rosedale, J. L., *Biochem. J.*, **19**, 1015 (1925).
7. Plimmer, R. H. A., and Rosedale, J. L., *Biochem. J.*, **19**, 1012 (1925).
8. Koehler, A. E., *J. Biol. Chem.*, **42**, 267 (1920).
9. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 109 (1929).
10. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
11. Calvery, H. O., *J. Biol. Chem.*, **83**, 631 (1929).
12. Folin, O., and Denis, W., *J. Biol. Chem.*, **12**, 245 (1912).
13. Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, **21**, 165 (1900).

ISOLATION OF METHIONINE BY ENZYMATIC HYDROLYSIS

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The latest addition to the list of amino acids is the sulfur-containing amino acid, methionine, isolated by Mueller (1) from casein, eggs, edestin, and wool. He ascribed to it the empirical formula, $C_5H_{11}SNO_2$. Barger and Coyne (2) from a study of its chemical structure were led to believe that the compound was γ-methiol-α-amino-n-butyric acid. This they substantiated by actual synthesis using the Zelinsky-Stadinoff modification of the Strecker method. This structure was confirmed by Windus and Marvel (3) who succeeded in synthesizing methionine by the malonic ester method. They furthermore resolved the inactive synthetic product into its optical isomers and showed that the synthetic levo compound was identical with the naturally occurring amino acid (4). A new method of synthesis has recently been worked out by Barger and Weichselbaum (5) in which a much larger yield of the amino acid is realized.

There was some question in Mueller's mind as to whether the compound he had isolated was an integral part of the protein molecule or whether it was a secondary decomposition product formed during the acid hydrolysis. He therefore fed the compound to find out if it were oxidizable in the animal body (6). He found that the sulfur of this amino acid was oxidized to sulfate. He also stated that he was able to isolate methionine from "aminoids," a commercial enzymatic hydrolysate of protein, although no details of this isolation were given. It is not entirely clear whether he carried out further hydrolysis or whether the compound was obtained by extraction of the aminoids. Neither the purity, yield, nor rotation of the product so obtained was given.

The isolation of the amino acid after enzymatic hydrolysis would offer conclusive proof that the compound was not formed during the acid hydrolysis from some other substance in the sense of a secondary split-product. The isolation in this manner would also eliminate the action of boiling concentrated acid on the methionine. The possibility existed at the time this work was instituted that the methionine isolated by acid hydrolysis might be partially racemized, accounting for the low rotation of methionine. It is known that boiling concentrated hydrochloric acid racemizes cystine (7), and it did not appear unlikely that the same might be true for methionine. Recently, however, Windus and Marvel (4) have resolved methionine obtaining l-methionine with a rotation of $[\alpha]_D^{25} = -8.1°$ and d-methionine with a rotation of $[\alpha]_D^{25} = +8.7°$. Mueller found a rotation of $-7.2°$ for the product he had isolated.

It is, of course, of interest to ascertain which amino acids are split off from the protein molecule by a given enzyme. For instance it has been shown that tyrosine is readily split off by trypsin, whereas proline and phenylalanine are seldom detected among its hydrolytic products (8). Furthermore, if methionine were split off by trypsin a method might be made available in which the bothersome contamination of phenylalanine could be circumvented. It will be remembered that Mueller had much difficulty with phenylalanine.

In the isolation of tryptophane from protein, tryptic hydrolysis of casein is resorted to. The tryptophane is precipitated from acid solution by mercuric sulfate. Methionine, if present, should be in the filtrate since Mueller (1) and Barger and Coyne (2) have shown that the mercuric salt of methionine is soluble in acid solution. In the tryptic hydrolysis of casein large amounts of tyrosine are also obtainable. From the preparative standpoint, the isolation of methionine might be advantageously combined with the isolation of tryptophane and tyrosine from casein if methionine were split off by the action of trypsin.

The isolation of methionine from casein hydrolyzed by trypsin was therefore undertaken. Tyrosine and tryptophane were isolated by the methods of Cox and King (9). Methionine was isolated by a modified method of Mueller (1) from the acid filtrate resulting from the mercuric sulfate precipitation of the tryptophane, demonstrating that methionine is hydrolyzed from the

protein molecule by trypsin. From 3 kilos of casein an average yield of 2 gm. of analytically pure methionine was obtained. Mueller reported a yield per pound of casein of 1.5 to 2.5 gm. of 75 to 90 per cent pure methionine. Barger and Coyne (2) using Mueller's method obtained 1 to 3 gm. per kilo with a purity of 85 to 95 per cent. Their yield of course of the final analytically pure material would naturally be considerably lower depending on the ease of removing the contaminating substance. In this laboratory the acid hydrolysis has given somewhat higher yields of methionine than were obtained by enzymatic hydrolysis, 1 to 2 gm. of pure methionine per kilo of casein being obtained.

<div align="center">EXPERIMENTAL</div>

1500 gm. of commercial casein are placed in a 5 gallon bottle and covered with about 8 liters of water. After the casein is thoroughly moistened, 150 gm. of sodium carbonate and 15 gm. of sodium fluoride dissolved in 2 liters of water are added. 50 gm. of pancreatin as a thin paste in 200 cc. of water are then added. The mixture is finally diluted to 15 liters, a layer of toluene added, the mixture thoroughly shaken, and kept in the warm room at approximately 37° for 2 weeks. Each day during this period the bottle is shaken. On the 5th day another 50 gm. of pancreatin are added. Two batches are always run at the same time and later in the process combined.

At the end of the digestion period the bottles are cooled in the ice box and the tyrosine and undissolved material filtered off. The tyrosine was isolated from this material by the method of Cox and King (9). The tryptophane was precipitated by $HgSO_4$ as described by Cox and King (9).

To the filtrate from the precipitation of tryptophane 1 kilo of $HgSO_4$ dissolved in 5 liters of 7 per cent H_2SO_4 is added. A cold saturated solution of sodium hydroxide is then added slowly with stirring until the solution reacts neutral to Congo red. The alkali must not be in excess. After standing overnight, the precipitate is filtered and washed with water four or five times, the precipitate being resuspended in water each time. This precipitate will be designated as Fraction I.

To the combined filtrate and washings 500 gm. of $HgSO_4$ dissolved in 5 liters of 5 per cent H_2SO_4 are added. The reaction

mixture is again carefully neutralized with NaOH to Congo red, filtered, and the precipitate washed.   This is Fraction II.

A third precipitation is made with 3 liters of 5 per cent H_2SO_4 containing 300 gm. of $HgSO_4$.   This fraction (Fraction III) is treated in the same manner as the other two.

The second batch is treated as above and Fractions I from both batches are combined, suspended in 3 liters of water, and a hot, concentrated solution of $Ba(OH)_2$ added with stirring until the mixture reacts faintly alkaline to litmus.   Sufficient finely pulverized $Ba(OH)_2$ is then added to give a 2 per cent solution.   The mixture is heated with stirring on the steam bath for 0.5 hour and filtered.   The residue is again suspended in 3 liters of water and solid $Ba(OH)_2$ added to make a 2 per cent solution, and heated as before.   Such an extraction is repeated three times more.

The filtrates from the above extractions are combined and concentrated *in vacuo* to a volume of 1 liter.   The solution is then freed quantitatively of barium or sulfate ions, made acid with HCl, and heated to boiling.   A boiling saturated aqueous solution containing 200 gm. of $HgCl_2$ is then added.   The mixture is allowed to stand in the ice box overnight and before filtering cooled further by placing in an ice-salt bath.

Fractions II and III from both batches are treated as above up to the concentration *in vacuo*.   Here the Fractions II and the Fractions III are each concentrated to 500 cc., combined, and after removal of barium and sulfate ions, 150 gm. of $HgCl_2$ are added to the solution acidified with HCl.

The mercuric salts from both of the above precipitations are combined after filtration and washing with ice water.   They are then suspended in 1 liter of water, 50 cc. of concentrated HCl added, and the solution shaken with H_2S.   The mercuric sulfide is then filtered off, washed with a little water, and the filtrate and washings evaporated under reduced pressure almost to dryness. The residue is dissolved in 95 per cent ethyl alcohol with warming on the steam bath and decolorized with norit.   To the cooled filtrate 35 cc. of aniline[1] are added and the mixture is allowed to stand in the ice box overnight.   After further cooling in an ice-

salt mixture the methionine is filtered.off. To purify the methionine, it is dissolved in a small volume of water, decolorized with norit, and precipitated by the addition of absolute alcohol. The yield of analytically pure methionine averaged about 2 gm. The optical rotation of the compound was found to be $[\alpha]_D^{25} = -7.3°$, practically the same as the rotation of methionine obtained by acid hydrolysis. Digestions were also carried out for 1 month instead of 2 weeks without materially improving the yield.

Analysis
3.152 mg. substance: 0.263 cc. N at 25° and 742 mm.
0.0461 gm.   "   : 0.0717 gm. BaSO$_4$
              C$_5$H$_{11}$O$_2$NS. Calculated. S 21.5, N 9.4
                          Found.   " 21.4, " 9.3

SUMMARY

It has been shown that methionine is split off from casein by tryptic digestion.

The rotation of methionine obtained by enzymatic hydrolysis is practically the same as the rotation of that obtained by acid hydrolysis.

The isolation of methionine has been combined with the isolation of tyrosine and tryptophane from casein hydrolyzed by trypsin. The methionine is found in the filtrate resulting from the precipitation of the tryptophane with mercuric sulfate in acid solution.

BIBLIOGRAPHY

1. Mueller, J. H., *J. Biol. Chem.*, **56**, 157 (1923).
2. Barger, G., and Coyne, F. P., *Biochem. J.*, **22**, 1417 (1928).
3. Windus, W., and Marvel, C. S., *J. Am. Chem. Soc.*, **52**, 2575 (1930).
4. Windus, W., and Marvel, C. S., *J. Am. Chem. Soc.*, **53**, 3490 (1931).
5. Barger, G., and Weichselbaum, T. E., *Biochem. J.*, **25**, 997 (1931).
6. Mueller, J. H., *J. Biol. Chem.*, **58**, 373 (1923–24).
7. Hoffman, W. F., and Gortner, R. A., *J. Am. Chem. Soc.*, **44**, 341 (1922).
8. Mitchell, H. H., and Hamilton, T. S., The biochemistry of the amino acids, New York, 214 (1929).
9. Cox, G. J., and King, H., in Clarke, H. T., Organic syntheses, **10**, 100 (1930).

THE BASIC AMINO ACIDS FROM NEUROKERATIN: IS NEUROKERATIN A TRUE KERATIN?*

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Ewald and Kühne in 1877 (1) first introduced the method of using the digestive juices for the preparation of material for histological examination. They observed that if sections of the various soft organs of the body were treated with gastric and pancreatic juices and then extracted successively with organic solvents, dilute acid, and alkali, "Nur ein Gewebe und zwar dasjenige, von dem es am wenigsten zu erwarten war, zeigte dieselbe erstaunliche Resistenz wie das Horn gegen unsere Lösungsmittel, nämlich das nervöse: die graue Substance des Ruckenmarkes und des Gehirns, sowie die Retina." In order to show that the material from human brain was not an artificial product, they varied the order of the procedure but apparently obtained the same product each time; like horn, it yielded more tyrosine and less leucine than other proteins and contained nitrogen, 2.93 per cent of sulfur, and 1.6 per cent of ash.

Neurokeratin was prepared in 1890 from human brains by Kühne and Chittenden (2) and was analyzed for carbon, hydrogen, nitrogen, sulfur, phosphorus, and ash. They were unable to obtain material of the same elementary composition in any two preparations. Argiris (3) employed only tryptic digestion in the procedure and also omitted the extraction with dilute alkali; while Nelson (4, 5) used repeated peptic digestion and a short treatment with alkali to prepare neurokeratin from human brain. The

analyses of the products obtained by each of these workers are shown in Table I.

Preparation of Neurokeratin

The following procedure was adopted after considerable preliminary study. The meninges and larger blood vessels were dissected away from 35 pounds of fresh ice-cold pig brains and the blood was removed by washing with warm water. The material was then ground and repeatedly extracted with acetone until all the soluble substances had been removed. The light yellow powder that was obtained was sifted to remove the blood vessels and other gross impurities, and ground again. The powder was extracted with absolute alcohol for 24 hours, the liquid being removed by pressure filtration, and the press-cake was extracted

TABLE I

Elementary Analysis of Neurokeratin

	Author	Kühne and Chittenden (2)	Argiris (3)	Nelson (5)
	per cent	per cent	per cent	per cent
Carbon	55.53	56.11–58.95	56.61	56.0 –58.0
Hydrogen	6.96	7.26– 8.02	7.46	7.25– 9.00
Nitrogen	14.1	11.46–14.32	14.17	11.5 –14.5
Sulfur	1.98	1.63– 2.24	2.27	1.38– 2.24

with dry ether for the same length of time. Alternate extraction with alcohol and with ether was continued as long as these solvents removed any appreciable amount of material. The press-cake was then pulverized and soaked in absolute alcohol for 2 weeks at 40°. After this treatment the cerebrosides, and other substances soluble in warm alcohol, were removed by extraction in a large Soxhlet apparatus for 28 days. The residual brain material no longer yielded substances soluble in hot or cold acetone, ethyl alcohol, ether, chloroform, or benzene. The product was then digested with 30 times its weight of 0.3 per cent hydrochloric acid containing 0.03 per cent of a 1:15,000 solution of pepsin (Fairchild). After 4 days the supernatant liquid was removed and fresh pepsin and hydrochloric acid were added. This process was repeated five times. The activity of the digesting solu-

tion was checked by the method of Gilman and Cowgill (6) and found to be satisfactory. At the completion of the peptic digestion, the residue was washed with 50 volumes of distilled water. The combined hydrochloric acid was carefully neutralized with sodium borate and the material was then suspended in 30 volumes of sodium borate-boric acid buffer solution at pH 7.8. Trypsin was added and digestion was allowed to proceed at 37° until there was no further change in the ratio of amino to total nitrogen in the filtrate. Toluene was used as a preservative. The brain residue was acidified with hydrochloric acid to pH 1 and the inorganic salts were removed by repeated extraction with 0.5 per cent hydrochloric acid. The neurokeratin was dried by alcohol and ether in the usual manner. It contained C 55.53, H 6.96, N 14.1, S 1.98, P 0.00, and ash 1.1 per cent.

TABLE II

Basic Amino Acids in Neurokeratin

	Author		Argiris (3)		Nelson (5)
	Analysis I	Analysis II	Analysis I	Analysis II	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Arginine (isolated as flavianate)	3.13	3.45	2.28	2.19	1
Arginine (corrected for solubility of arginine silver).........	3.85	4.08			
Histidine.......................	1.75	1.56	Not isolated		3
Lysine.........................	3.13	1.76	2.72	2.68	8

Basic Amino Acids in Neurokeratin

Argiris (3) reported the basic amino acid content of neurokeratin, as determined by the Kossel-Kutscher method, to be arginine 2.28 per cent, from the weight of the copper nitrate double salt, and lysine 2.72 per cent, based on the weight of the crude picrate (Table II). He was unable to isolate histidine. Nelson (5) analyzed a little less than 3 gm. of neurokeratin by the Van Slyke method and obtained results indicating 1 per cent of arginine, 3 per cent of histidine, and 8 per cent of lysine.

Analysis I—The basic amino acids were isolated from 48.0 gm. (corrected) of neurokeratin by the method described by Vickery

and Block (7). Arginine flavianate equivalent to 3.13 per cent was obtained; corrected for the solubility of arginine silver in 6 liters of solution, this is equivalent to 3.85 per cent of arginine. Histidine diflavianate equivalent to 1.75 per cent of histidine and lysine picrate of decomposition point 264° equivalent to 3.13 per cent of lysine were also isolated.

Analysis II—From a smaller amount of neurokeratin (17.3 gm.), arginine was isolated as the flavianate in an amount equivalent to 3.45 per cent; when corrected for the solubility of arginine silver in 3 liters of solution, this was equal to 4.08 per cent of arginine. The arginine flavianate contained 6.6 per cent of sulfur, theory 6.56 per cent. Histidine was isolated as the diflavianate equivalent to 1.56 per cent; the preparation contained 8.21 per cent of sulfur, theory 8.17 per cent. Lysine was isolated as the picrate of decomposition point 264° in an amount equivalent to 1.76 per cent of the protein.

<div align="center">CONCLUSION</div>

The sample of neurokeratin described in this paper is similar to the classical preparations both in elementary and in basic amino acid composition. Although the solubility relationships of this material suggest that it should be classified with the keratins, it shows little or no similarity to the true keratins either in the proportions of basic amino acids yielded (8) or in the molecular ratios of these bases to each other. The true keratins yield histidine, lysine, and arginine in such molecular proportions that these amino acids are in the ratios of approximately 1:4:12 (8), while the ratios of the molecular proportions of histidine : lysine : arginine yielded by this specimen of neurokeratin, calculated from the highest values found in these analyses, were 1:2:2. *On the basis of the experimental results reported in this paper, it may be concluded that neurokeratin is not a true keratin.*

BIBLIOGRAPHY

1. Ewald, A., and Kühne, W., *Verhandl. Naturhist.-med. Vereins Heidelberg,* **1,** 457 (1877).
2. Kühne, W., and Chittenden, R. H., *Z. Biol.,* **26,** 291 (1890).
3. Argiris, A., *Z. physiol. Chem.,* **54,** 86 (1907).
4. Nelson, B. E., *Psychiat. Bull., New York State Hosp.,* **9,** 1 (1916).
5. Nelson, B. E., *J. Am. Chem. Soc.,* **38,** 2558 (1916).
6. Gilman, A., and Cowgill, G. R., *J. Biol. Chem.,* **88,** 743 (1930).
7. Vickery, H. B., and Block, R. J., *J. Biol. Chem.,* **93,** 105 (1931).
8. Block, R. J., and Vickery, H. B., *J. Biol. Chem.,* **93,** 113 (1931).

THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI

XXVI. SEPARATION OF THE LIPOID FRACTIONS FROM THE LEPROSY BACILLUS*

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INTRODUCTION

The causative agent of leprosy was first recognized by Hansen (1) in Norway who isolated the leprosy bacillus in 1873. The organism was studied by Neisser (2) and he determined that it was acid-fast. The bacillus was successfully cultivated by Clegg (3) in the Philippines in 1909 and shortly afterwards by Duval (4) in Louisiana. Duval (5) succeeded in producing typical leprous lesions in the monkey by the injection of massive doses of the artificially cultivated bacillus.

A review of the chemistry of the leprosy bacillus is given by Long (6) but it is evident from a perusal of the literature that only fragmentary information exists concerning the chemical composition of this organism. This lack of knowledge is surprising because leprosy has been a dreaded disease in all parts of the world from the earliest times to the present.

The staining properties of the fatty substances obtained from the leprosy bacillus by extraction with various solvents together with the acid fastness of the extracted bacilli were studied by microchemical methods by Unna, Jr. (7), while the first macro-

chemical investigation of the lipoids was reported by Gurd and Denis (8) who used the organism that had been isolated by Duval.

These investigators found that the dry bacillus yielded about 34 per cent of fat together with a small quantity of a waxy solid containing phosphorus and which was regarded as lecithin.   It was further found that the fat contained unsaturated fatty acids and a pigment which was classified as a lipochrome.   After the fat had been saponified, a small amount of unsaponifiable matter was obtained which gave color reactions similar to those of cholesterol.

Long and Campbell (9) in a comparative study of the lipids of acid-fast bacteria found that the leprosy bacillus gave 9.7 per cent of total lipid.   The lipid had a saponification number of 188 and contained 27.2 per cent of unsaponifiable matter.   Other investigations dealing with the metabolism of *Bacillus lepræ* have been published by Long (10) and by Reed and Rice (11).   According to Kendall, Day, and Walker (12) various acid-fast bacteria including *Bacillus lepræ* produce soluble lipase.

Since leprosy and tuberculosis are closely related diseases, it was decided to include the leprosy bacillus in the program of the cooperative chemical and biological investigation on tuberculosis which is being conducted under the auspices of the Medical Research Committee of the National Tuberculosis Association. Such a comparative study might reveal significant differences in chemical composition and in biological reactions of the lipoid fractions obtained from two closely related organisms which differ in virulence or pathogenicity.

In the extraction and separation of the lipoid fractions from *Bacillus lepræ* we have followed the methods which have been developed in this laboratory in connection with investigations on the tubercle bacilli (13).   We were naturally anxious to secure the lipoid material in a condition as similar as possible to that in which it existed in the living cells.   We therefore employed the mildest methods of extraction, using cold alcohol and ether followed by chloroform, and the extracts were concentrated at a temperature not exceeding 40°.

Through the cooperation of the Medical Research Committee of the National Tuberculosis Association and the Mulford Biological Laboratories, Sharp and Dohme, we were provided with a very large quantity of living *Bacillus lepræ*.   The bacilli were

extracted with large quantities of alcohol and ether and then with chloroform.

The alcohol-ether extract was separated into phosphatide, acetone-soluble fat, and a small amount of wax. From the aqueous solution which remained after the ether and alcohol had been evaporated we isolated a considerable amount of polysaccharide. The chloroform extract yielded a large amount of wax on the evaporation of the solvent. All of these compounds were similar in properties to corresponding fractions obtained from tubercle bacilli, but they were much more highly pigmented.

Biological experiments are under way to determine the reactions produced by the lipoids of the *Bacillus lepræ* and the results will be reported separately.

<div align="center">EXPERIMENTAL .</div>

The *Mycobacterium lepræ* used in this work is known as the Hygienic Laboratory Strain No. 370 (Apa case) and it was isolated from a case of human leprosy in Honolulu about 1909. The organism has been carried in the Mulford Biological Laboratories at Glenolden, Pennsylvania, since February 4, 1926, and is there known as Strain 1629. The bacillus grows well on a synthetic medium and is highly chromogenic.

For the present work we were provided with 3000 cultures of *Bacillus lepræ* and this is the largest quantity of acid-fast bacteria that we have ever worked up at one time. The cultures were grown for 6 weeks in 1 liter Pyrex bottles, each bottle containing 200 cc. of the Long synthetic medium (14). The living bacilli were collected on large Buchner funnels, washed with water, and immediately placed in a mixture of equal parts of alcohol and ether contained in 5 gallon Pyrex bottles.

During the filtration streams of carbon dioxide were passed over the funnels; the solvents used for extraction had been saturated with carbon dioxide. In all the subsequent operations that will be described below, air was always displaced by carbon dioxide. All solvents were freshly distilled and saturated with carbon dioxide before they were used. The alcohol had been distilled over potassium hydroxide.

It will be mentioned here and not referred to again that all of the bacterial extracts were filtered through Chamberland candles

under carbon dioxide pressure before the lipoids were isolated. The filtrates thus obtained were brilliantly clear and we believe that all cell debris had been removed.

Extraction with Alcohol-Ether

The bacteria were equally distributed between seven 5 gallon bottles. The containers were securely stoppered, thoroughly shaken, and transported to the Sterling Chemistry Laboratory. The bottles were shaken occasionally for about 1 week; the bacterial cells were then allowed to settle and the clear, deep reddish colored supernatant extract was siphoned off with carbon dioxide pressure. The bacterial residues were distributed evenly between four 5 gallon Pyrex bottles and again extracted two times in a similar manner, 5 liters of ether and 1 liter of alcohol being used for each bottle. The bacteria were finally filtered on Buchner funnels and washed with ether. The alcohol-ether extracts and washings were combined and worked up, as will be described later.

Extraction with Chloroform

Two extractions with chloroform were carried out in the following manner. The bacterial residues were returned to the four large bottles, 4 liters of chloroform being added to each bottle, and the mixtures were shaken occasionally for about 1 week. Bacterial suspensions in chloroform do not settle and it is necessary therefore to recover the extract by filtration on Buchner funnels. After filtering, the treatment with chloroform was repeated and the mixtures were again filtered. The bacterial residues were finally shaken with a mixture of 4 liters of ether and 1 liter of alcohol for the purpose of removing the chloroform which adhered to the cells. After filtering and washing with the alcohol-ether mixture, the bacterial cells were dried *in vacuo* at 40° and reserved for the isolation of water-soluble constituents such as proteins, carbohydrates, nucleic acid, etc. The dried bacterial residue formed a chocolate-colored crumbly mass weighing 3389.8 gm.

Chloroform-Soluble Wax

The last alcohol-ether extract mentioned above was concentrated to dryness, when a waxy residue was obtained which weighed 23.3 gm., and this was combined with the main lot of the

chloroform-soluble material. The chloroformic solutions were evaporated to dryness under reduced pressure, leaving a deep reddish waxy residue. The material constitutes the crude wax; the total weight was 444.8 gm. It was analyzed for phosphorus and nitrogen but only traces of these elements were found, thus indicating that the wax fraction was practically free from phosphatide. The material is reserved for future investigations.

Examination of the Alcohol-Ether Extract

The extract was perfectly clear and of deep reddish color. The ether was removed at a temperature of 35–40° by a current of carbon dioxide and the alcohol was distilled off under reduced pressure at the temperature mentioned above, carbon dioxide being admitted through the capillary tube. The dark red lipoid material which remained in the aqueous suspension was extracted with ether. The aqueous solution was reserved for the isolation of a polysaccharide, as will be described later.

Separation of the Acetone-Soluble Fat

The ethereal extract was dried with sodium sulfate, filtered, washed, and was then concentrated by a current of carbon dioxide to a volume of 2 liters. An equal volume of acetone was added, when a dark red oily mass was precipitated. The clear supernatant liquid was decanted and concentrated until most of the ether had been removed. More acetone was added and the solution was cooled in ice water and the precipitate which separated was filtered off and washed with cold acetone. The acetone solution was concentrated under reduced pressure, yielding a dark red oily fat which did not solidify entirely on cooling. A further small quantity of a similar fraction was recovered in purifying the phosphatide and it was combined with the main lot, giving a total of 289.5 gm. of fat soluble in cold acetone.

Purification of the Phosphatide

The crude acetone-insoluble material which was separated during the isolation of the acetone-soluble fat was precipitated repeatedly from ethereal solution by acetone. The crude phosphatide, which was finally obtained by pouring the ethereal solution into cold methyl alcohol, was a bright red amorphous powder. In

working up the mother liquors from these precipitations the ether was first removed by distillation in a current of carbon dioxide. The resulting acetone solution was cooled in cracked ice, when a considerable quantity of wax-like material was precipitated as a nearly white amorphous powder and was removed by filtration. The final acetone mother liquor yielded on concentration a small amount of fat which, as mentioned above, was combined with the acetone-soluble fat.

The crude phosphatide which weighed 100.5 gm. could not be freed from the adhering pigment by precipitation with acetone. The product was still bright red after it had been precipitated twenty times from ether with acetone.   The use of methyl alcohol in place of acetone was more effective in removing the coloring matter, and by precipitating the phosphatide five times from chloroform with methyl alcohol it was obtained as a straw-colored amorphous powder.   In order to obtain the phosphatide in powder form it was necessary to pour the chloroformic solution into cold methyl alcohol; when the process was reversed a sticky salve-like mass was obtained.

The purified phosphatide melted with decomposition at 231°. On analysis it gave 7.36 per cent of ash, 1.75 per cent of phosphorus, and a barely measurable trace of nitrogen.

Separation of Crude Polysaccharide

The aqueous alcoholic solution which remained after the lipoids had been extracted was concentrated under reduced pressure to a volume of about 400 cc.   An aqueous solution of 20 per cent lead acetate was added until no further precipitation occurred, and the precipitate after it had been filtered and washed with water was discarded.   The filtrate and washings were combined, concentrated under reduced pressure, and precipitated by adding an excess of basic lead acetate and ammonia.   The heavy white precipitate was filtered off after the mixture had stood overnight and was washed with water.   It was then suspended in water, decomposed with hydrogen sulfide, and the lead sulfide was filtered off and washed with water.   The clear filtrate was concentrated *in vacuo* to a syrup and the latter was dried in a vacuum desiccator. The thick syrup was dehydrated by grinding in a mortar under absolute alcohol until a nearly white amorphous powder was ob-

tained. After this substance had been dried in a vacuum desiccator, it weighed 41.2 gm. The substance gave no reduction when boiled with Fehling's solution but after it had been boiled for some time with dilute sulfuric acid it gave a heavy reduction with Fehling's solution. It is evidently a polysaccharide similar to corresponding fractions obtained from the other acid-fast bacteria (13).

A summary of the various products isolated from the leprosy bacillus is given in Table I and for comparison we also include similar fractions isolated from the human tubercle bacillus.

The most notable difference in the lipoids of the two organisms is the lower phosphatide and wax content of the *Bacillus lepræ*. It must be remembered, of course, that the values given in Table I

TABLE I

Substances Isolated from Bacillus

	Bacillus lepræ 3000 cultures		Human tubercle bacillus, Strain H-37 2000 cultures	
	gm.	*per cent*	*gm.*	*per cent*
Phosphatide......................	100.5	2.25	253.1	6.54
Acetone-soluble fat...............	289.5	6.47	240.0	6.20
Chloroform-soluble wax...........	444.8	9.98	427.0	11.03
Total lipoids.....................	834.6	18.70	920.1	23.78
Polysaccharide...................	41.2	0.92	33.9	0.87
Dry bacillary residue.............	3389.8	80.38	2902.0	75.01
" bacterial matter per culture...	1.488		1.928	

do not represent the total lipoids of the bacillus but only those portions that can be extracted by alcohol-ether and by chloroform at room temperature.

The bacterial residue which had been defatted as described above still contained a large amount of lipoid material. Some 3 or 4 per cent of lipoids could be removed by prolonged grinding of the defatted bacteria in a ball mill with alcohol and ether, but when some of the defatted bacteria was refluxed for 4 hours with a mixture of equal parts of alcohol and ether containing 1 per cent of hydrochloric acid, we obtained 30 per cent of soluble material of which more than one-half or 17 per cent of the bacteria represented ether-soluble lipoids and 13 per cent was soluble in water. It is

evident therefore that the leprosy bacillus contained over 30 per cent of total lipoids.

BIBLIOGRAPHY

1. Hansen, G. A., *Virchows Arch. path. Anat.*, **79**, 32 (1880).
2. Neisser, A., *Breslau. ärztl. Z.*, No. 20, 21 (1879); *Virchows Arch. path. Anat.*, **84**, 514 (1881).
3. Clegg, M. T., *Philippine J. Sc.*, Sect. B., **4**, 403 (1909).
4. Duval, C. W., *J. Exp. Med.*, **12**, 649 (1910).
5. Duval, C. W., *J. Exp. Med.*, **13**, 374 (1911).
6. Wells, H. G., DeWitt, L. M., and Long, E. R., The chemistry of tuberculosis, Baltimore, 110 (1923).
7. Unna, P., Jr., in Unna, P. G., Dem Hamburger Dermatologen, Dermatologische Studien, Hamburg and Leipsic, **21**, pt. 2, 283 (1910).
8. Gurd, F. B., and Denis, W., *J. Exp. Med.*, **14**, 606 (1911).
9. Long, E. R., and Campbell, L. K., *Am. Rev. Tuberc.*, **6**, 636 (1922–23).
10. Long, E. R., *Am. Rev. Tuberc.*, **5**, 857 (1921–22).
11. Reed, G. B., and Rice, C. E., *J. Bact.*, **16**, 97 (1928).
12. Kendall, A. I., Day, A. A., and Walker, A. W., *J. Infect. Dis.*, **15**, 439 (1914).
13. Anderson, R. J., *J. Biol. Chem.*, **74**, 525 (1927).   Anderson, R. J., and Roberts, E. G., *J. Biol. Chem.*, **85**, 509, 529 (1929–30).   Chargaff, E., Pangborn, M. C., and Anderson, R. J., *J. Biol. Chem.*, **90**, 45 (1931).
14. Long, E. R., *Am. Rev. Tuberc.*, **13**, 393 (1926).

TRYPTOPHANE METABOLISM

II. THE GROWTH-PROMOTING ABILITY OF dl-TRYPTOPHANE

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INTRODUCTION

In nutrition experiments involving the use of free amino acids, it is ordinarily assumed that the optical antipode of the naturally occurring enantiomorph can be utilized only with difficulty, if at all. Therefore, when a racemic modification of the amino acid is employed, it is customary to insure the presence of an adequate amount of the naturally occurring isomer by feeding the racemic mixture at twice the level optimum for the naturally occurring active form. The justification for this procedure seems to be based primarily upon findings recorded by several investigators that, when racemic amino acids are given in doses sufficient to overwhelm the mechanism of the normal organism for disposing of them, they may be partially recovered in the urine. Optical analysis of the portion excreted invariably shows it to be composed either exclusively, or for the greater part, of the optically active form not occurring naturally.

Observations of this nature have been made by Wohlgemuth (1905) on inactive leucine, aspartic acid, glutamic acid, and tyrosine. The findings on dl-leucine have been confirmed by Abderhalden and Samuely (1906); on dl-tyrosine by Dakin (1910) and Kotake, Matsuoka, and Okagawa (1922). Similar studies with essentially similar results have been made on dl-alanine (Hirsch (1905); Plaut and Reese (1906); Schittenhelm and Katzenstein (1906); Brugsch and Hirsch (1906, 1907); Oppenheimer (1907); Abderhalden and Schittenhelm (1907); Blum (1920); Abderhalden and Franke (1928)). Friedmann (1908) failed to recover administered dl-alanine but did recover some administered dl-

norleucine.   Inactive phenylalanine has likewise been tested
by Dakin (1909) and by Kotake, Masai, and Mori (1922).   The
latter investigators used the levo, racemic, and dextro forms and
found that whereas the levo form was almost completely oxidized,
somewhat less than two-thirds of the dextro isomer was destroyed.
The oxidation of the racemic modification was intermediate in
extent. ˙ Abderhalden and Weil (1912) fed *dl*-histidine to rabbits as
a means of resolving it, the levo form being the more readily
destroyed in the body.

It is obvious, therefore, that the unnatural component of a
racemic modification of an amino acid is less readily attacked in
the body than is the naturally occurring isomer.   Whether this
difference in susceptibility to attack is sufficient to render effective
utilization of an optical isomer impossible when only small
amounts of its antipode are required (as, for example, in growth)
may well be questioned.

From a purely theoretical consideration the conversion of an
optically active amino acid into its antipode in the animal body,
although the process may be limited, does not seem impossible.
Kotake, Masai, and Mori (1922) report that not only *l*-phenyl-
alanine, but also *d*- and *dl*-phenylalanine, can be oxidatively
deaminized in the body to form phenylpyruvic and *p*-hydroxy-
phenylpyruvic acids.   In a recent study involving the administra-
tion of smaller amounts of *l*- and *dl*-phenylalanine, Shambaugh,
Lewis, and Tourtellotte (1931) have obtained evidence of the
excretion of considerable amounts of phenylpyruvic acid.   The
results were apparently unaffected by the optical properties of the
phenylalanine administered.   Kotake and Okagawa (1922) iso-
lated approximately the same amount of *p*-hydroxyphenylpyruvic
acid from the urine of rabbits given like amounts of *l*- and *dl*-
tyrosine.   Waser (1923) found only the intermediary oxidative
products in the urine after administering *dl*-tyrosine to dogs.
Evidence of the conversion of a ketonic acid into the corresponding
amino acid was first adduced by Knoop and Kertess (1911).   More
recently Harrow and Sherwin (1926) have shown that 4-imidazole
pyruvic acid is capable of replacing histidine in the diet, as have
also Jackson (1929) and Berg, Rose, and Marvel (1929–30) that
3-indolepyruvic acid may serve in lieu of required dietary trypto-
phane. ˙ It is possible, of course, that the physiological intercon-

version of optical antipodes may occur directly, without intermediate deaminization. Inversion through the corresponding α-hydroxy acids is, likewise, another possibility.

Apparently the replaceability of a naturally occurring amino acid in the diet by the corresponding racemic form has not been extensively investigated. McGinty, Lewis, and Marvel (1924–25) record one experiment in which growth on d-lysine was somewhat greater than on an equal amount of the dl form. They seem to emphasize, not the degree of utilization, but the fact that utilization of the dl-lysine can occur. Several other investigators have fed the racemic modification of other amino acids at twice the level of the naturally occurring form as a matter of precaution.

It seemed worth while, therefore, in continuing our studies on tryptophane metabolism, to determine how efficiently the rat may utilize the racemic form of tryptophane for growth. No work concerning the nutritive value of dl-tryptophane has been published. Matsuoka, Takemura, and Yoshimatsu (1925) obtained considerably lower yields of kynurenic acid on administering dl-tryptophane to rabbits than on giving the levo form. The apparent failure of the animal organism to convert the dextro enantiomorph into kynurenic acid does not indicate that this isomer might not be utilized for growth. Interpretations of kynurenic acid production studies made thus far incline one toward the opinion that kynurenic acid is not an intermediary metabolite of tryptophane but rather an end-product (Kotake and Ichihara, 1931). Probably the metabolic path involved in the utilization of tryptophane for maintenance and growth is distinct from the one by which kynurenic acid is produced (Homer, 1915). Hence one must use caution in applying interpretations of studies made on kynurenic acid production to other aspects of tryptophane metabolism. The vast difference in amount of tryptophane administered in kynurenic acid and in growth studies (usually 25 to 50 times as much in the former as in the latter cases) challenges the propriety of using results from one type of study as a basis for prediction concerning the other.

<div align="center">EXPERIMENTAL</div>

For an accurate comparison between tryptophane-deficient diets supplemented with l-tryptophane and similar diets supple-

mented with *dl*-tryptophane, it is imperative that the additions
be no larger than the minimum required for good growth.   Inas-
much as we have heretofore found that good growth could be ob-
tained on 20 mg. of tryptophane fed separately each day or on a
dietary inclusion of 0.2 per cent, we chose these as the maximum
supplements.   In the first series of experiments the rates of growth
were established on diets containing varying suboptimum supple-
ments of *l*-tryptophane.   *dl*-Tryptophane was then substituted for
the levo form at twice these levels.   If the dextro enantiomorph
were not utilizable, the animals should continue to grow at the
same rate because they would still be receiving the same supple-
ment of *l*-tryptophane.   Should the dextro enantiomorph be
utilizable, either partially or wholly, an accelerated growth rate
would result.   To test this proposition twenty rats from three
litters were divided into five groups of four.   The rats were housed
in individual false bottomed cages.   The basal diet, fed *ad libitum*,
consisted of acid-hydrolyzed casein[1] 14.7, cystine 0.3, starch 40,
sucrose 15, Crisco 19, cod liver oil 5, salt mixture[2] 4, and agar 2 per
cent.   Each animal received twice daily 100 mg. of yeast vitamin
Harris in the form of a pill containing also an equal weight of
starch.   During the first part of the experiment, all of the rats
received the tryptophane supplement twice daily in the vitamin
pill, Group 1 receiving no supplement; Group 2, 1.25 mg.; Group
3, 2.5 mg.; Group 4, 5 mg.; and Group 5, 10 mg. in each pill.   Be-
cause these groups did not show sufficient differences in growth
rate (for reasons discussed later), the tryptophane was introduced,
after 32 days, into the food mixture, displacing an equal amount of
acid-hydrolyzed casein.   The groups now received 0.0, 0.025, 0.05,
0.1, and 0.2 per cent of *l*-tryptophane, respectively.   Even though
calculations based on food consumption data show that less trypto-
phane was being consumed, a somewhat more satisfactory response
was obtained during this second period.   After 16 days on the
above diets, two of the rats from each group (excepting from
Groups 1 and 5, which served only as controls) were placed on diets
containing twice as much of the *dl*-tryptophane as was contained
of the levo form in the previous diets.   The other two rats re-
mained on the *l*-tryptophane diets throughout.

[1] See Berg, C. P., and Rose, W. C., *J. Biol. Chem.*, **82**, 480 (1929).

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

All of the active tryptophane was prepared in this laboratory according to the Cox and King (1930) method. The specific rotations of the preparations used in these studies ranged from $[\alpha]_D^{20}$ = $-33.0°$ to $-33.6°$ in 0.5 per cent aqueous solution. These values are in good agreement with those recorded in the literature (Hopkins and Cole (1903), $-33°$, solvent not specified, but presumably water; Fischer (1908), $-29.75°$ to $40.3°$ in approximately 0.5 per cent water solution, temperature not specified; Abderhalden and Baumann (1908), $-30.33°$ at 20° in 0.5 per cent aqueous solution). The dl-tryptophane was prepared by refluxing a 2 per cent solution of l-tryptophane in 10 per cent barium hydroxide until the isolated and recrystallized tryptophane exhibited no optical activity. At least 80 hours refluxing was usually allowed. Recovery of the tryptophane from the baryta solution was accomplished by cautiously adding sulfuric acid until tests for barium and sulfate ion were both negative, filtering off the barium sulfate, concentrating the solution in vacuo to crystallize out the tryptophane, and recrystallizing the isolated amino acid from alcohol. We are convinced from our observations thus far that free tryptophane is considerably more difficult to racemize than is usually supposed and we are investigating this angle of the question somewhat more in detail. The nitrogen content of the l and dl preparations checked well with the theoretical.

Since the data secured on Litter 1 are typical for the entire lot of rats of this series, we are economizing space by presenting growth curves (Chart I) and food consumption records (Table I) of this litter only, using Rat 38 from Litter 3 as a control receiving the maximum supplement of tryptophane throughout. The growth curve and food consumption data of this rat are typical of the group on 0.2 per cent l-tryptophane.

The data presented indicate several interesting facts. Apparently a tryptophane-deficient diet supplemented with yeast vitamin Harris, instead of with yeast (see Berg, Rose, and Marvel (1929–30)) as a source of vitamin B is not improved by the addition of 0.025 per cent l-tryptophane to the extent that it can produce growth. Loss of weight does not occur quite as rapidly, however, as it does on the tryptophane-deficient diet. If 0.05 per cent l-tryptophane be fed instead of 0.025 per cent as a supplement to such a diet, the rat maintains weight or grows at a very slow rate.
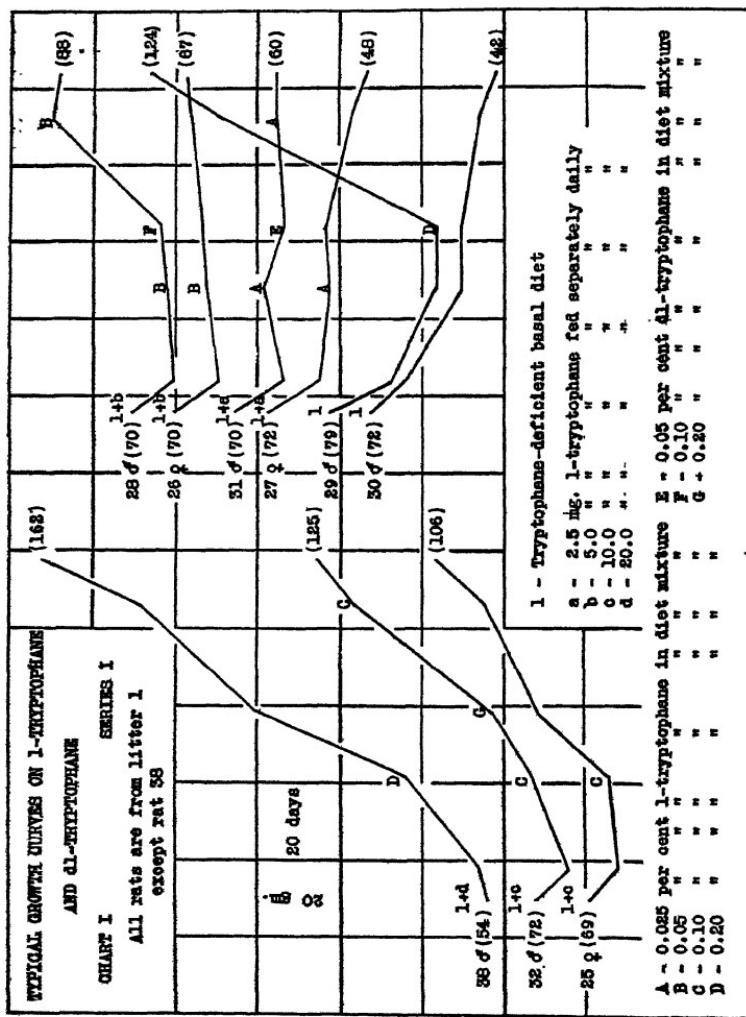
CHART I. Growth curves. Initial and final weights are given in parentheses

The most pronounced effects are noted on diets supplemented with 0.1 per cent or 0.2 per cent of the amino acid.   Growth is better

TABLE I

Food Consumption and Body Weight Changes

| Rat No. and sex | Days | Average daily | | Diet* | Rat No. and sex | Days | Average daily | | Diet* |
		Change in weight	Food consumption				Change in weight	Food consumption	
		gm.	*gm.*				*gm.*	*gm.*	
38 ♂†	1– 8	+0.25	3.1	1 + d					
	9–32	+0.75	4.0	1 + "					
	33–48	+2.25	5.5	D					
	49–76	+1.00	5.7	"					
	77–88	+2.00	6.3	"					
32 ♂	1– 8	−1.00	5.0	1 + c	25 ♀	1– 8	−0.88	4.3	1 + c
	9–32	+0.38	4.1	1 + "		9–32	+0.09	2.9	1 + "
	33–48	+0.56	4.0	C		33–48	+1.06	3.0	C
	49–76	+1.21	5.6	G		49–76	+0.50	4.8	"
	77–88	+0.75	4.0	C		77–88	+1.00	5.0	"
28 ♂	1– 8	−1.25	3.9	1 + b	26 ♀	1– 8	−1.38	4.5	1 + b
	9–32	+0.09	2.6	1 + "		9–32	+0.15	3.4	1 + "
	33–48	+0.06	2.3	B		33–48	+0.06	2.7	B
	49–76	+0.96	3.6	F		49–76	+0.11	2.7	"
	77–88	−0.17	3.4	B		77–88	+0.06	3.0	"
31 ♂	1– 8	−1.38	4.8	1 + a	27 ♀	1– 8	−1.50	4.5	1 + a
	9–32	+0.17	3.0	1 + "		9–32	−0.13	3.2	1 + "
	33–48	−0.31	2.0	A		33–48	+0.06	2.3	A
	49–76	+0.07	1.9	E		49–76	−0.21	1.9	"
	77–88	0.00	1.9	A		77–88	−0.50	2.7	"
29 ♂	1– 8	−1.88	4.5	1	30 ♂	1– 8	−1.13	4.3	1
	9–32	−0.46	2.1	1		9–32	−0.54	1.7	1
	33–48	0.00	1.8	1		33–48	0.00	1.8	1
	49–76	+1.86	5.0	D		49–76	−0.14	1.3	1
	77–88	+1.58	5.0	"		77–88	−0.33	1.0	1

* For the significance of the diet designations see Chart I.

† All rats except Rat 38 are from the same litter.

when tryptophane is included in the ration at these latter levels than when fed separately at levels of 10 and 20 mg. per day, respec-

tively, even though the daily tryptophane ingestion is considerably less in these latter instances, as shown by calculations based on daily food consumption.   The reason for this is undoubtedly that the simultaneous presence of tryptophane and the other amino acids necessary for anabolism insures a more complete retention of tryptophane than could be expected when that amino acid is fed alone in comparatively large doses.   In the latter case much of the excess tryptophane undoubtedly is catabolized.   The curves of Rats 28 and 32, as compared with those of Rats 26 and 25 respectively, indicate quite strikingly that more than the levo component of the racemic mixture can be utilized for growth.

Inasmuch, however, as one cannot estimate fairly from the above experiments how well the *dl*-tryptophane compares with the levo form in promoting growth, we undertook a second series designed to answer this phase of the question more adequately.   The plan differed essentially in that the animals receiving a given amount of *l*-tryptophane during the control period were allowed the same amount of the racemic modification during the experimental period, rather than a double portion.   Hence, comparisons on the same basis were possible.   Twelve rats from two litters were divided into six pairs.   One pair from each litter served as controls receiving no tryptophane.   One of the rats in the second pair from each litter received 0.1 per cent *dl*-tryptophane in the diet; the other, 0.1 per cent *l*-tryptophane.   After 40 days the administration of tryptophane was reversed, the first rat now receiving 0.1 per cent *l*- and the other, 0.1 per cent *dl*-tryptophane.   The administration of tryptophane to the third pair from each litter followed the same plan, excepting that the amount of tryptophane included in the diet was twice as great in each case.   Typical growth curves shown in Chart II indicate that the average rate of weight increment of the animal receiving the *dl*-tryptophane is practically the same as that of the control on the same amount of the naturally occurring active tryptophane.   Rats 43 to 48 are from Litter 6; Rats 49 to 52 from Litter 7.   Food consumption data and average weight increments are given in Table II.

Food consumption data in Tables I and II show that rats consume more food when the diet is supplemented with tryptophane, whether that supplement be fed separately or incorporated directly into the *ad libitum* mixture.   The rats receiving the different

supplements of tryptophane (Table I) consumed approximately equal amounts of the tryptophane-deficient food mixture during the initial 8 days, but altered the intake thereafter; those receiving

GROWTH CURVES ON 1-TRYPTOPHANE
AND dl-TRYPTOPHANE

CHART II   SERIES II

C - 0.1 per cent 1-tryptophane
D - 0.2 "   "   "   "
F - 0.1 per cent dl-tryptophane
G - 0.2 "   "   "   "

20 gm.

20 days

(232)

(202)

(235)

(171)

(152)

(155)

(213)

F

C

F

(84)

D

G

C

46♀ (65)

47♀ (67)

C

52♂ (60)

51♂ (71)

C

(84)

43 ♂ (58)

44 ♂ (61)

D

G

F

48 ♀ (55)

45 ♂ (71)

1

50 ♂ (62)

49 ♂ (62)

G

D

D

†(42)
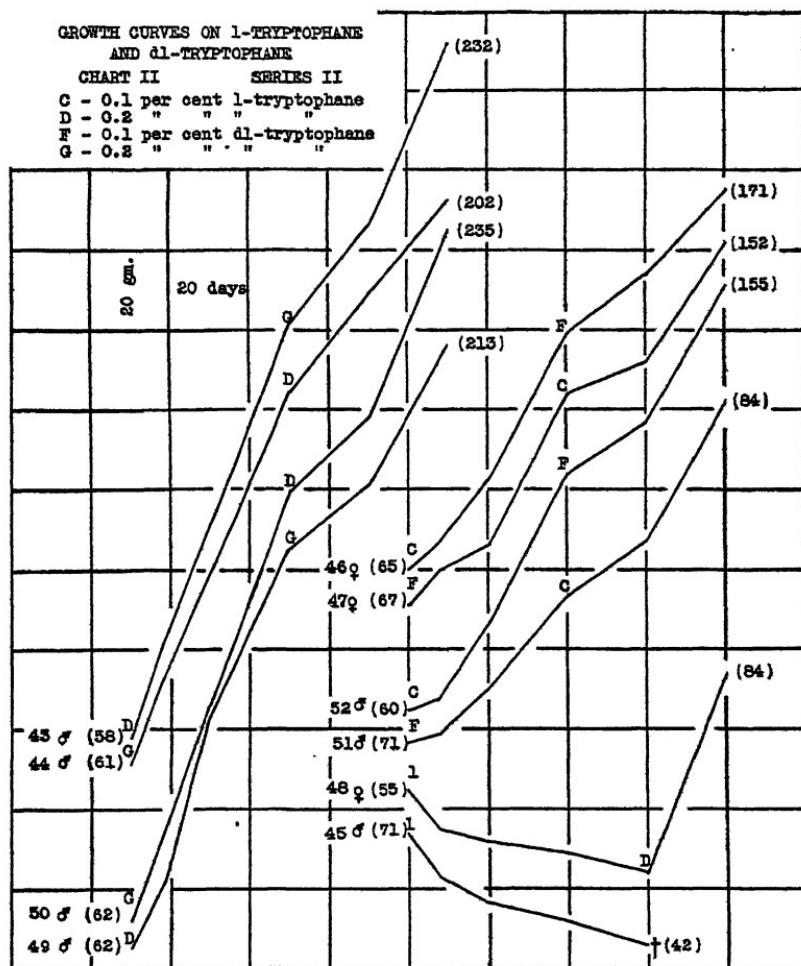
CHART II. Growth curves. Initial and final weights are given in parentheses.

no supplement showed a sharply diminished intake which continued to decrease with loss in body weight; those receiving increasing supplements of tryptophane showed progressively less tendency

to diminish the initial intake.   With increased rate of growth, food
consumption is increased.   In order to acquire data based on food
consumption, we started a series of rats on limited food intake.
These data are not now being reported, but they convince us that

TABLE II

Food Consumption and Body Weight Changes

| Rat No. and sex | Days | Average daily | | Total gain | Efficiency quotient | Tryptophane supplement |
		Increase in weight	Food consumption			
		gm.	*gm.*	*gm.*		*per cent*
43 ♂	1–40	2.58	8.1	103	2.80	0.2 (*l*)
	41–80	1.53	9.3	61	3.15	0.2 (*dl*)
44 ♂	1–40	2.33	7.9	93	3.10	0.2 "
	41–80	1.20	9.1	48	3.92	0.2 (*l*)
49 ♂	1–40	2.50	8.4	100	2.90	0.2 "
	41–80	1.28	8.6	51	3.62	0.2 (*dl*)
50 ♂	1–40	2.68	8.8	107	2.85	0.2 "
	41–80	1.65	9.1	66	2.77	0.2 *l*
46 ♀	1–40	1.48	6.9	59	5.25	0.1 "
	41–80	1.18	8.5	47	4.99	0.1 (*dl*)
47 ♀ *	1–40	0.93	5.3	37	7.07	0.1 "
	41–80	1.20	8.1	48	5.55	0.1 (*l*)
51 ♂	1–40	1.33	6.7	·53	5.45	0.1 (*dl*)
	41–80	0.95	7.7	38	5.88	0.1 (*l*)
52 ♂	1–40	1.48	6.4	59	5.15	0.1 "
	41–80	0.90	7.7	36	6.28	0.1 (*dl*)

* During the first few weeks of the experiment blood was frequently
found in the cage of Rat 47.   Data on this rat are, therefore, excluded from
further consideration.

paired feeding methods (Mitchell and Beadles, 1929–30) or
similar methods based on limited intakes of food cannot be used to
advantage in comparing diets practically devoid of an essential
nutritive component such as tryptophane with adequate diets.
It seems that more satisfactory results might be obtained if animals

receiving a deficient diet were forced, rather, to consume and retain amounts of food comparable to those naturally eaten by animals receiving an adequate diet, as has been done in vitamin studies by Marrian, Baker, Drummond, and Woollard (1927). At any rate, we feel from our preliminary study that methods based on limited food intake are best adapted to comparisons of diets which would naturally be consumed in amounts adequate, or more than adequate, for maintenance. Jackson (1929) has reached a similar conclusion. In instances in which we fed the 0.2 per cent *l*-tryptophane diet to animals which had previously consumed voluntarily the same amount of tryptophane-deficient diet, there was no improvement outside of checked loss of weight or perhaps a very negligible weight increment; however, when a similar change was tried on rats which had established a food consumption level

TABLE III

Comparison of Average Efficiency Quotients

Rat No.	*l*-Tryptophane, 40 days		*dl*-Tryptophane, 40 days	
	Per cent	Efficiency quotient	Per cent	Efficiency quotient
43 ♂, 44 ♂, 49 ♂, 50 ♂	0.2	3.10	0.2	3.18
46 ♀, 51 ♂, 52 ♂	0.1	5.43	0.1	5.57

on a diet containing 0.1 per cent *l*-tryptophane, an appreciable change in growth rate was observed for a short period of time.

Because it seemed to us that there would be less tendency to mask minor differences in growth-promoting ability if the impulse to eat were kept unrestrained, we chose to permit our rats in Series II to consume the diet *ad libitum* and to evaluate the data on the basis of food consumption by utilizing the efficiency quotient of Palmer and Kennedy (1931). This quotient represents the digestible dry matter consumed in gm. per gm. of gain in weight per 100 gm. of body weight. In cases in which gains in weight are made, the smaller quotient represents the more efficient utilization of food for growth. Should the quotient be applied to an animal losing weight, it becomes negative in sign and increases numerically with diminishing losses. Inasmuch as all rats in Series II received the same diet, except with respect to the optical form of the trypto-

phane included, we have disregarded the digestibility of the dry matter consumed.   Inasmuch, also, as each rat was fed on both the *l*-tryptophane and *dl*-tryptophane, each for the same period of time, it seemed unnecessary to establish a preliminary quotient. Individual quotients, thus modified, give the numerical evaluations for each period recorded in Table II.   That these efficiency quotients verify the findings indicated by growth curves is shown in the comparison of average efficiency quotients, Table III.   The difference in average efficiency quotients of the rats on *dl*-tryptophane and of those on the levo form at the same level is so slight as to be negligible.

SUMMARY

Rats fed *ad libitum* on a tryptophane-deficient diet were able to maintain weight or grow very slowly when 0.05 per cent *l*-tryptophane was added to the food mixture.   Supplements of 0.025 per cent *l*-tryptophane were inadequate for growth.   Inclusions of 0.1 per cent and 0.2 per cent *l*-tryptophane permitted fair rates of growth, the latter considerably better than the former.

When the *l*-tryptophane supplements were replaced by double the amounts of the racemic modification the rats showed pronounced increase in rate of growth, indicating that more than the naturally occurring component was utilized.

An evaluation of data secured on rats receiving *l*-tryptophane and those receiving the *dl* form in like amount showed no significant differences either in growth or in efficiency quotients calculated from food consumption and growth records.

It is therefore concluded that *dl*-tryptophane possesses nearly, if not quite the same ability to supplement a tryptophane-deficient basal diet for the purpose of growth as does the naturally occurring levo form.

BIBLIOGRAPHY

Abderhalden, E., and Baumann, L., *Z. physiol. Chem.*, **55**, 412 (1908).

Abderhalden, E., and Franke, K., *Fermentforschung*, **10**, 39 (1928).

Abderhalden, E., and Samuely, F., *Z. physiol. Chem.*, **47**, 346 (1906).

Abderhalden, E., and Schittenhelm, A., *Z. physiol. Chem.*, **51**, 323 (1907).

Abderhalden, E., and Weil, A., *Z. physiol. Chem.*, **77**, 435 (1912).

Berg, C. P., Rose, W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 219 (1929–30).

Blum, W., *Beitr. Physiol.*, **1**, 385 (1920); abstracted in *Chem. Zentr.*, **3**, 392 (1920).

Brugsch, T., and Hirsch, R., *Z. exp. Path. u. Therap.*, **3**, 638 (1906); **4**, 947 (1907).

Cox, G. J., and King, H., in Clarke, H. T., Organic syntheses, **10**, 100 (1930).

Dakin, H. D., *J. Biol. Chem.*, **6**, 235 (1909); **8**, 25 (1910).

Fischer, H., *Z. physiol. Chem.*, **55**, 74 (1908).

Friedmann, E., *Beitr. chem. Physiol. u. Path.*, **11**, 151 (1908).

Harrow, B., and Sherwin, C. P., *J. Biol. Chem.*, **70**, 683 (1926).

Hirsch, R., *Z. exp. Path. u. Therap.*, **1**, 141 (1905).

Homer, A., *J. Biol. Chem.*, **22**, 391 (1915).

Hopkins, F. G., and Cole, S. W., *J. Physiol.*, **29**, 451 (1903).

Jackson, R. W., *J. Biol. Chem.*, **84**, 1 (1929).

Knoop, F., and Kertess, E., *Z. physiol. Chem.*, **71**, 252 (1911).

Kotake, Y., and Ichihara, K., *Z. physiol. Chem.*, **195**, 171 (1931).

Kotake, Y., Masai, Y., and Mori, Y., *Z. physiol. Chem.*, **122**, 195 (1922).

Kotake, Y., Matsuoka, Z., and Okagawa, M., *Z. physiol. Chem.*, **122**, 166 (1922).

Kotake, Y., and Okagawa, M., *Z. physiol. Chem.*, **122**, 201 (1922).

Marrian, G. F., Baker, L. C., Drummond, J. C., and Woollard, H., *Biochem. J.*, **21**, 1336 (1927).

Matsuoka, Z., Takemura, S., and Yoshimatsu, N., *Z. physiol. Chem.*, **143**, 199 (1925).

McGinty, D. A., Lewis, H. B., and Marvel, C. S., *J. Biol. Chem.*, **62**, 75 (1924–25).

Mitchell, H. H., and Beadles, J. R., *J. Nutr.*, **2**, 225 (1929–30).

Oppenheimer, S., *Beitr. chem. Physiol. u. Path.*, **10**, 273 (1907).

Palmer, L. S., and Kennedy, C., *J. Biol. Chem.*, **90**, 545 (1931).

Plaut, M., and Reese, H., *Beitr. chem. Physiol. u. Path.*, **7**, 425 (1906).

Schittenhelm, A., and Katzenstein, A., *Z. exp. Path. u. Therap.*, **2**, 560 (1906).

Shambaugh, N. F., Lewis, H. B., and Tourtellotte, D., *J. Biol. Chem.*, **92**, 499 (1931).

Waser, E., *Helv. Chim. Acta*, **6**, 206 (1923).

Wohlgemuth, J., *Ber. chem. Ges.*, **38**, 2064 (1905).

THE PLANT COLORING MATTER, ROBININ*

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In the course of an investigation dealing with the spectral transmittancies of flavonols and their glucosides, the writer isolated and purified a quantity of robinin from the white flowers of the common locust (*Robinia pseudoacacia*, L.). It was thought that the composition of this material would agree with that represented by the formula $C_{33}H_{42}O_{20} \cdot 8H_2O$, which is given for robinin by Perkin and Everest[1] (2) in their treatise on the natural organic coloring matters.

Although Perkin and Everest appear to accept the formula mentioned above, they also refer to a report in Russian of an investigation by Waliaschko (3) in which $C_{33}H_{40}O_{19} \cdot 7\frac{1}{2}H_2O$ is given as the formula for robinin. It is entirely possible that in 1918 Perkin and Everest were unaware of the existence in German (4) (1904) of practically a duplicate of the Russian paper mentioned above and for this reason failed to attach to Waliaschko's work the significance which it merited. At any rate, they mention his work only briefly and in such a manner as to convey the impression that Waliaschko at the time of his researches must

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[1] A reference has been made to the fact that robinin was first isolated by Zwenger and Dronke (1) from the flowers of white azalea instead of from the flowers of false acacia, which is one of the common names of *Robinia pseudoacacia*. Azaleas are generally shrubs and are easily distinguished from the locust since the latter rarely occurs as a shrub, but is a large tree sometimes reaching a maximum height of 80 feet. It is a tree with a very rough bark and white fragrant flowers having standards yellowish at the base. Britton and Brown give the following common names for *Robinia pseudoacacia*: locust tree, false or bastard acacia called also white, yellow, black, red, and green locust, honey locust, pea-flower locust, and post locust.

not have been cognizant of Perkin's previous work (1902) on robinin (5). Although Perkin may have arrived at this conclusion as a result of his perusal of the Russian account, it is quite apparent from the German report that Waliaschko was familiar with Perkin's previous researches and moreover considered his results erroneous in several important respects.

The results of my investigation, which was undertaken to determine the correct formula, confirm those reported by Waliaschko. Accordingly, pure air-dried robinin possesses the formula, $C_{33}H_{40}O_{19} \cdot 7\frac{1}{2}H_2O$, and on hydrolysis with acids yields 2 molecules of rhamnose, 1 molecule of galactose, and 1 molecule of the free flavonol coloring matter, $C_{15}H_{10}O_6$, which is undoubtedly kaempferol, as Perkin has shown.

Interesting is the fact, which has not been reported by other investigators, that robinin occurs in two mutually convertible modifications, and may, therefore, be considered as representing a case of polymorphism. One form, crystallized from water, melts at 195–197°, whereas the other, crystallized from ethyl alcohol, melts at 249–250°.

EXPERIMENTAL

The sample of robinin employed in this investigation was obtained from the white flowers of common locust (*Robinia pseudoacacia*, L.), which were collected in June from a single large tree located near Washington, D. C. The air-dried flowers, after being coarsely ground, were exhaustively extracted in a large extractor of the Soxhlet type first with u. s. p. ether to remove fats and other ether-soluble substances and then with redistilled 95 per cent ethyl alcohol, which removed the coloring matter. The alcoholic extract was filtered and evaporated to a small volume, the final traces of alcohol being removed by vacuum distillation. Water was added to the syrupy residue, and the resulting liquid was shaken with successive portions of ether which were discarded. After this treatment, the aqueous liquid deposited overnight a quantity of semicrystalline robinin. This deposit was collected on a Buchner funnel, washed with water, and crystallized from water, dilute pyridine, dilute acetic acid, and finally with water.

Thus obtained, robinin consists of microscopic clusters of pale

viridine yellow crystals which somewhat resemble rutin in appearance, although robinin forms more loosely arranged clusters which are composed of larger individual crystals. My sample of robinin began to sinter about 190° and melted at 195–197° to a thick amber-colored liquid which darkened on further heating. Zwenger and Dronke (1) who first isolated this coloring matter in 1861, stated their preparation sintered at 190° and melted at 195°. Perkin claims that his product sintered at 190° and melted at 196–197°, whereas Waliaschko's product sintered at 188° and melted at 195°. In general, my sample of robinin agreed in other properties with those reported first by Zwenger and Dronke and later verified by Perkin and Waliaschko.

When robinin, crystallized from water and melting in the anhydrous state at 195–197°, is partly or entirely dissolved by boiling with absolute or 95 per cent ethyl alcohol and then allowed to crystallize, its melting point changes to 249–250°. The high melting modification will revert to the form giving the low melting point when recrystallized from water. The possibility that the material lacked chemical homogeneity was, of course, considered. It was rejected by analyzing the alcohol-insoluble (Fraction A) and alcohol-soluble (Fraction B) portions, which were obtained by boiling a sample of robinin with a quantity of alcohol insufficient to dissolve the whole sample and subsequently recrystallizing the two fractions from water and drying at 115°.

Fraction A.   0.1776 gm.: 0.3471 gm. CO_2 and 0.0878 gm. H_2O
          0.1864 " : 0.3638 · " " " 0.0928 " "
Fraction B.   0.1954 " : 0.3818 " " " 0.0964 " "
          0.1759 " : 0.3442 " " " 0.0857 " "
      Found.   Fraction A.   C 53.30, 53.23; H 5.53, 5.57
        "   "   B. " 53.29, 53.37; " 5.52, 5.45
    $C_{33}H_{40}O_{19}$ requires C 53.49, H 5.44
    $C_{33}H_{42}O_{20}$ (Perkin's formula) requires C 52.22, H 5.58

The optical and crystallographic properties of the two robinin modifications determined by G. L. Keenan, of the Food and Drug Administration, United States Department of Agriculture, are given below.

α-Robinin, which was crystallized from hot water and melted in the anhydrous state at 195–197°, consists of rods with square ends. These show the refractive indices $n_\alpha = 1.490$ (length-

wise), n_β = 1.612 (crosswise), both ± 0.003, and n_γ = 1.702 ± 0.005 (crosswise); $n_\gamma - n_\alpha$ = 0.212 ± 0.004. In parallel polarized light with crossed nicols, the extinction is parallel, and the elongation is negative. In convergent polarized light, the substance is biaxial, but interference figures are rare.

β-Robinin, which was crystallized from 95 per cent or absolute alcohol and melted at 249–250°, consists of rods with square ends. These show the refractive indices n_α = 1.600 (crosswise) and n_γ = 1.620 (lengthwise); $n_\gamma - n_\alpha$ = 0.020, all ± 0.003. In parallel polarized light with crossed nicols, the extinction is parallel, and the elongation is positive. In convergent polarized light, the substance is biaxial, although interference figures are rare. Anomalous interference colors, peculiar blues and purples, are characteristic for this material when examined with crossed nicols.

Water of Crystallization—Determinations were made of the water of crystallization contained in robinin crystallized from water and dried at ordinary room temperature. A sample exposed to room air for 4 days after crystallization gave the following results.

0.7414 gm. (heated to 105–110° *in vacuo*) lost 0.1194 gm. H_2O
0.1056 " : 0.1731 gm. CO_2 and 0.0601 gm. H_2O
0.1188 " : 0.1951 " " " 0.0676 " "
        Found. H_2O 16.10; C 44.70, 44.79; H 6.37, 6.37
        $C_{33}H_{40}O_{19} \cdot 8H_2O$ requires H_2O 16.29, C 44.77, H 6.38

A sample exposed to room air for at least 10 days after crystallization gave the following results.

0.2104 gm. (heated to 115°) lost 0.0328 gm. H_2O
0.2200 " ("   " 115°) " 0.0336 " "
0.1488 " : 0.2469 gm. CO_2 and 0.0816 " "
        Found. H_2O 15.59, 15.27; C 45.25; H 6.14
        $C_{33}H_{40}O_{19} \cdot 7\frac{1}{2}H_2O$ requires H_2O 15.42, C 45.23, H 6.33

It is evident, therefore, that robinin crystallizes with 8 molecules of water, but that $\frac{1}{2}$ molecule is very easily lost at ordinary room temperature. The more stable compound appears to be the one retaining $7\frac{1}{2}$ molecules of water. Even this compound loses another $\frac{1}{2}$ molecule of water on long standing, as has been shown by Waliaschko, who made a determination on the air-dried compound after 2 years and obtained figures corresponding to 7 molecules of water of crystallization.

Hydrolysis of Robinin—Upon hydrolysis robinin is resolved into 1 molecule of kaempferol, 1 molecule of galactose, and 2 molecules of rhamnose, according to the equation

$$C_{33}H_{40}O_{19} + 3H_2O = C_{15}H_{10}O_6 + C_6H_{12}O_6 + 2C_6H_{12}O_5$$

Perkin has definitely proved that the free flavonol pigment obtained by hydrolysis of robinin is kaempferol, and Waliaschko has identified the two sugar components as rhamnose and galactose.

A portion of robinin was boiled 2 hours with approximately 1 per cent sulfuric acid. After hydrolysis, the mixture was set aside overnight. The insoluble portion was then collected and washed thoroughly with cold water, and dried at 130°.

0.6799 gm. robinin: 0.2627 gm. kaempferol
        Found. Kaempferol, $C_{15}H_{10}O_6$, 38.64
        $C_{33}H_{40}O_{19}$ requires kaempferol, $C_{15}H_{10}O_6$, 38.64

Analysis of the free flavonol pigment, dried at 130°, without subsequent recrystallization (Fraction A) and after recrystallization from dilute alcohol (Fraction B), gave results which agreed with those calculated for kaempferol.

Fraction A. 0.1893 gm.: 0.4344 gm. CO_2 and 0.0567 gm. H_2O
    "   B. 0.0995 " : 0.2293 "   "   " 0.0357 "   "
           0.1346 " : 0.3089 "   "   " 0.0455 "   "
        Found. C 62.58, 62.85, 62.59; H 3.35, 4.01, 3.78
        $C_{15}H_{10}O_6$ requires C 62.92, H 3.52

SUMMARY

An investigation of robinin, a coloring matter in the flowers of *Robinia pseudoacacia*, has shown that the anhydrous substance possesses the formula $C_{33}H_{40}O_{19}$ and that the air-dried material, after crystallization from water, contains 8 molecules of water of crystallization, ½ molecule of which is lost when the material stands at ordinary room temperature for approximately 10 days. As these findings are in agreement with the results of Waliaschko, the formula given in Perkin and Everest's treatise on the natural organic coloring matters is apparently incorrect and should be changed.

Robinin may be resolved into two mutually convertible modifications. These have been tentatively designated as *α*- and *β*-

robinin. The α form is obtained by crystallizing from water and dehydrating. It melts at 195–197°. The β form is obtained by crystallizing the α modification from absolute or 95 per cent ethyl alcohol. It melts at 249–250°. The β form may be converted to the α form by recrystallizing from water and dehydrating. Optical-crystallographic examinations have been made of the two dimorphic forms.

BIBLIOGRAPHY

1. Zwenger, C., and Dronke, F., *Ann. Chem. u. Pharm.*, suppl. 1, 257 (1861).
2. Perkin, A. G., and Everest, A. E., The natural organic colouring matters, London and New York, 181 (1918).
3. Waliaschko, N., *J. Russ. Physic.-Chem. Soc.*, **36,** 421 (1904).
4. Waliaschko, N., *Arch. Pharm.*, **242,** 383 (1904). Schmidt, E., *Arch. Pharm.*, **242,** 210 (1904).
5. Perkin, A. G., *J. Chem. Soc.*, **81,** 473 (1902).

BLOOD AND URINE STUDIES FOLLOWING BROMIDE INJECTION

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INTRODUCTION

The first paper (1) of this series discussed the response made by the mammalian organism to the sudden intravenous injection of hydrochloric acid. The present paper discusses the response made to the sudden intravenous injection of sodium chloride and sodium bromide. The purpose of the experiments was to determine: (1) the effect of increasing the chloride concentration in the blood on the distribution of chloride and bicarbonate between serum and cells; (2) the effect of increasing the chloride concentration in the blood on the urinary chloride excretion; (3) the effect of increasing the bromide concentration in the blood on the distribution of chloride and bromide between cells and serum; (4) the effect of increasing the bromide concentration in the blood on the urinary chloride and bromide excretion.

The work was especially stimulated by the observations of Hastings and van Dyke (2, 3) that the oral administration of bromides leads to an anomalous distribution of bromide in the blood of dogs and often produces edema. Odaira (4) showed that hypertonic saline infusions reduced the carbon dioxide content of plasma. Isotonic saline or sodium bromide infusions increased the carbon dioxide content. Hanzlik, De Eds, and Tainter (5) showed that the intravenous injection of 10 per cent saline caused a fall in the pH of arterial blood, but that isotonic or hypotonic saline did not regularly give the fall in pH.

Methods

The technique of injection of solution and sampling of blood and the methods for the determination of pH, total carbon dioxide, water, and refractive index were the same as were employed in the preceding paper (1). Where the percentage of cells was determined, the Van Allen hematocrit was used.

In Experiments XXIV and XXV the chlorides were determined by the wet ashing method of Van Slyke (6). In all the other experiments the chlorides and bromides were determined electrometrically with a silver electrode. It was found by comparing results on diluted urine and on protein-free urine that the diluted urine gave accurate results, but in the electrometric titration of serum and cells, tungstic acid filtrates made by the method of Folin and Wu were necessary. To 5 cc. of serum were added 41 cc. of water, 2 cc. of 10 per cent sodium tungstate, and 2 cc. of $\frac{2}{3}$ N sulfuric acid. The mixture was filtered, and 5 cc. of the filtrate were diluted with 45 cc. of water and titrated with 0.01 N silver nitrate. In the case of cells, the weight (approximately 1.0 gm.) was multiplied by 0.9185 to give the volume. For each unit of volume, 15.6 cc. of water were added. After thorough hemolysis, 1.7 cc. of the sodium tungstate solution per unit of volume and 1.7 cc. of sulfuric acid solution were added drop by drop. The mixture was filtered and 15 cc. of the resultant filtrate, diluted with 35 cc. of water, were used for titration. Urine samples were collected by catheterization between the drawing of the blood specimens.

Calculations

Calculations of bicarbonate, carbonic acid, and carbon dioxide tensions were made as in the preceding paper. The calculations from the wet ashing method were made in the usual manner. For the electrometric results, a correction had to be applied to the amount of bromide obtained by titration because of the presence of chloride. Thus if 3.0 cc. of 0.01 N silver nitrate were used to titrate the bromide and x cc. in addition to titrate the chloride, the corrected bromide value would be 2.8 and the chloride would be $x + 0.2$ cc. The correction factors were determined by Mr. J. E. Davis and Dr. S. K. Liu and are shown in Fig. 1.

Results

Effect of Injection of Sodium Chloride and Sodium Bromide on Blood

Seven injection experiments were performed including four bromide injection experiments (Nos. XIX to XXII) and three chloride injection experiments (Nos. XXIII to XXV). The
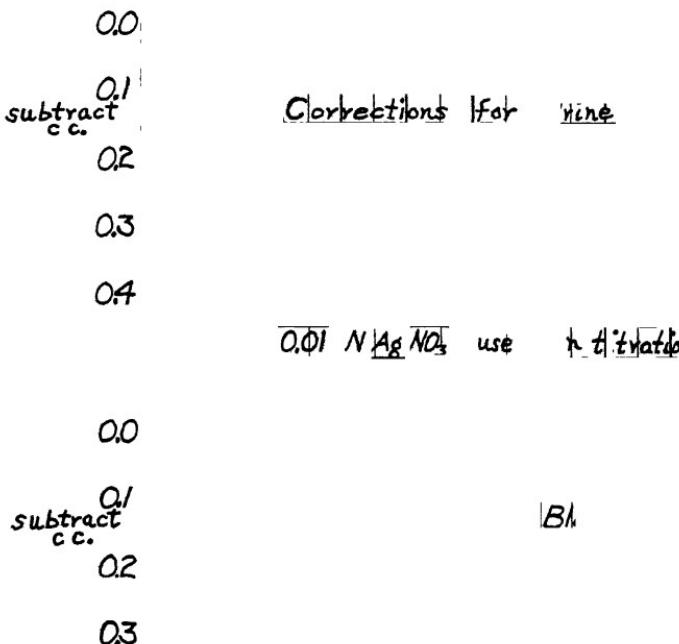
FIG. 1. Electrometric titration corrections for bromides in the presence of chlorides. The abscissæ represent the cc. of 0.01 N silver nitrate used in the titration and the ordinates represent the number of cc. to be subtracted from this value to give the corrected value. The upper line represents the corrections used for urine, the lower those used for blood.

protocols of two representative experiments of each group are included. Since the results of chloride and bromide are somewhat comparable they will be discussed together. In all cases there was a very marked fall in the refractive index with a gradual return toward normal. In one case of a bromide injection this return went well above normal. This is especially remarkable

when it is noted from the control experiments of the previous paper (1) that this could not be the effect of hemorrhage or anesthesia. The percentage of cells by the hematocrit reading showed a tremendous drop and in the recovery went far above normal in both chloride and bromide injection experiments. The water changes were marked and uniform, the serum water rising markedly and the cell water falling markedly with returns toward normal in both cases. These changes are more marked than in the acid injection experiments in the previous paper (1) because in the latter the ionic and dilution effects were opposed. The pH changes are not very marked. In the case of the bromide injections the tendency is toward a slight acidosis of hardly greater magnitude than was encountered in the control experiments (1). In one chloride injection an increase of 0.15 pH was observed.

Total carbon dioxide analyses were performed only in two of the chloride injection experiments. The results agree in the two cases. There was a slight reduction in total carbon dioxide, bicarbonate, and carbon dioxide tension of cells and serum. The bicarbonate ratio between cells and serum showed a fall. This is the opposite from the effects of acid injection. Several factors are concerned in these changes. Because of the impermeability of the erythrocytes to cations the addition of hypertonic sodium chloride to the serum would (1) lower the bicarbonate concentration by dilution, (2) cause the migration of chloride into the cells and bicarbonate from the cells to restore the Donnan equilibrium, and (3) increase the osmotic pressure of the serum with subsequent passage of water from cells to serum.

The results of the bromide and chloride analyses form the interesting part of these experiments. Experiments XXIII and XXIV as shown in Tables I and III illustrate that while the chloride rose in both serum and cells followed by a return toward normal, the distribution ratio remained constant. In the sodium bromide injections (Tables V and VII) the ratios did not remain so constant. The following are the most important results of the bromide injections. (1) After reaching a maximum at the end of injection, the serum bromides fell steadily. The cell bromides fell steadily, but in two of the four experiments started to rise again toward the end. The bromide distribution ratios between cells and serum showed a uniform steady rise. (2) Both the cell

and serum chlorides fell to a minimum at the end of injection and then after beginning to rise showed a late drop. Variations in the chloride ratios between cells and serum were slight. (3) The distribution ratio of the total halide in the cells to the total halide in the serum showed a uniform steady rise.

All of these changes seem to point to one thing; namely, that the red blood cells have an especial attraction for bromides. The chloride, bromide, and total halide distribution ratios of Experi-

TABLE I

Experiment XXIII. Sodium Chloride Injection

June 1, 1928; dog weight, 18 kilos; 8 gm. of barbital, ether during operation; 180 cc. of 2.0 N sodium chloride; death, $9\frac{1}{4}$ hours after injection.

Sample No.	Time	Amount of blood		Index of refraction	Hematocrit	H$_2$O		pH, colorimetric	[Cl]	$\frac{[Cl]_c}{[Cl]_s}$
		cc.				*gm. per cc.*	*gm. per gm.*		*mM per kg. H$_2$O*	
1	−8 min.	40	Serum	1.3491		0.936		7.36	124.4	0.740
			Cells		0.464		0.660		92.2	
2	+3 "	70	Serum	1.3430		0.961		7.37	207.4	0.740
			Cells		0.265		0.576		153.4	
3	15 "	55	Serum	1.3453		0.952		7.29	176.4	0.753
			Cells		0.356		0.619		132.9	
4	3 hrs.	40	Serum	1.3482		0.940		7.32	161.9	0.762
			Cells		0.472		0.636		123.4	
5	9 "	40	Serum	1.3485		0.940		7.34	164.0	0.749
			Cells		0.528		0.644		122.9	

ment XX are plotted against the pH in Fig. 2. It is seen that the bromide ratios are a uniform distance above the theoretical distribution curve calculated from the Van Slyke, Wu, and McLean equation. Similarly the total halide ratios correspond to the theoretical curve. The initial chloride ratio, represented by the open square, is the same as the total halide ratios at the same pH. This agrees with the result obtained for the chloride ratios in the previous paper of this series (1) where hydrochloric acid was injected. However, in the presence of bromide, the chloride distribution ratios, represented by the solid circles, are a rather uniform distance below the theoretical line. Two conclusions

may be drawn from these results. (1) The change in the bromide, chloride, and total halide distribution ratios with changing pH is in the direction and of a magnitude predicted by the application of the Gibbs-Donnan law to the system serum-cells. (2) It has been observed in our experiments and in those of
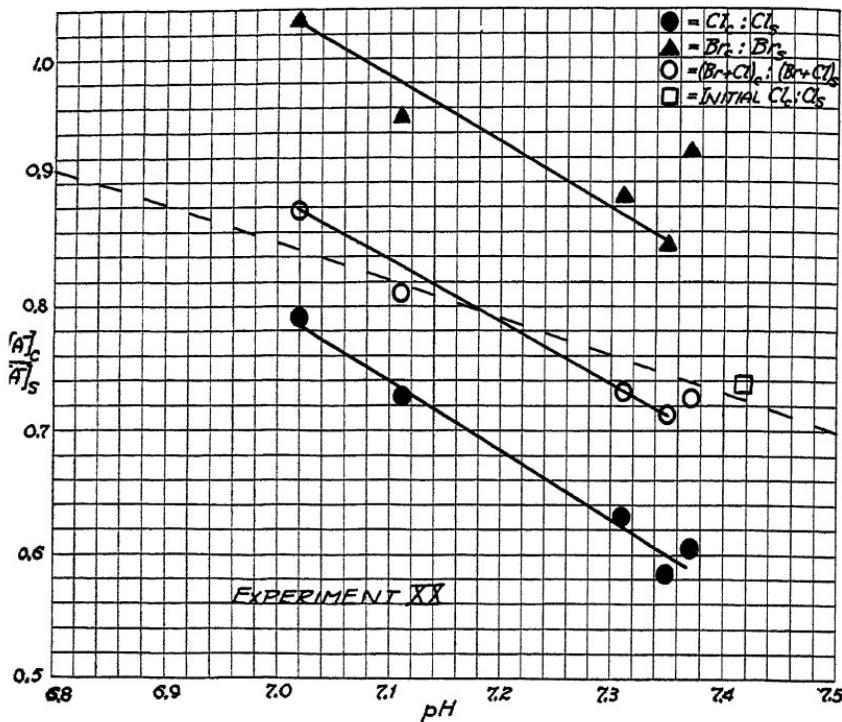
FIG. 2. Halide distribution ratios in Experiment XX. The abscissæ represent the pH and the ordinates the halide distribution ratio between cells and serum. The broken line running downward across the figure represents the theoretical distribution ratios calculated from the Van Slyke, Wu, and McLean formula.

Hastings and van Dyke (2, 3) that the distribution ratio $\frac{[Cl]_c}{[Cl]_s}$ did not fall below the value 0.5 at pH 7.4. This was the value found by Van Slyke, Hastings, Murray, and Sendroy (7) for the ratio $\frac{[H^+]_s}{[H^+]_c}$ at pH 7.4. If this figure is correct, 0.5 should be the

activity ratio of other monovalent diffusible ions. It is possible that under ordinary conditions, a certain proportion of the chloride of cells is not in ionic form, but that upon the addition of bromide, this non-ionic chloride is replaced by bromide, thus resulting in a chloride distribution in keeping with the hydrogen ion activity ratio. Until direct determinations of chloride activities are made, however, this must be regarded as merely a working hypothesis. This would indicate that the bromide would never completely replace the chloride in the red cells, but only enough to equalize the difference in activities.

TABLE II

Experiment XXIII. Sodium Chloride Injection. Analysis of Urine

Total chloride excretion = 126 mM, or 35 per cent of amount injected.

Sample No.	Time	Amount	Index of refraction	Specific gravity	Sugar	Albumin	[Cl]	Total Cl	Urine per min.	Cl per min.
	min.	*cc.*					*mM per l.*	*mM*	*cc.*	*mM*
1	Control	71	1.3491	1.040	Negative	Negative	251.0			
2	5	18	1.3422		+	+	296.0	5.4	3.6	1.06
3	13	57	1.3369	1.013	Negative	Negative	273.5	15.4	7.7	2.10
4	20	37	1.3372	1.015	"	Trace	296.0	11.1	4.9	1.45
5	30	50	1.3373	1.015	Trace	Negative	318.0	16.0	5.0	1.60
6	90	144	1.3385	1.018	Negative	Trace	373.0	51.8	2.4	0.89
7	180	55	1.3420	1.027	+	+	420.5	23.1	0.9	0.38
8	555	7	1.3543		+	++	415.5	2.9	0.02	0.01

Effect of Injection of Sodium Chloride and Sodium Bromide on Urine

Unlike the injection of hydrochloric acid, the injection of either sodium chloride or sodium bromide produced a marked diuresis. In Tables II, IV, VI, and VIII, the column of time denotes the number of minutes from the start of the injection to the end of that particular sample of urine. The samples were taken continuously and in order. In all experiments the injection of halide reduced the refractive index and specific gravity and increased the cc. of urine per minute. After a return to normal, the last samples had indices of refraction and specific gravities higher than normal with a diminished secretion per minute. An occasional

TABLE

Experiment XXIV. Sodium Chloride Injection

28, dog gh 24 kilos; 3.3 gm. of bar   100 cc. of 1.0 N sodium chloride.

Sample No.	Time	Amount of blood, cc.		Index of refraction	H_2O gm. per cc.	H_2O gm. per gm.	pH Colorimetric	pH Electrometric	Total $[CO_2]$ mM per kg. H_2O	$[BHCO_3]$ mM per kg. H_2O	$[H_2CO_3]$ mM per kg. H_2O	CO_2 tension mm. Hg	$[Cl]$ mM per kg. H_2O	$\frac{[HCO_3]_c}{[HCO_3]_s}$	$\frac{[Cl]_c}{[Cl]_s}$	$\frac{rCl}{rHCO_3}$
1	−10 min.	40	Serum	1.3511	0.937		7.23	7.38	23.40	22.15	1.245	37.8	104.4	0.904	0.815	0.901
			Cells			0.672			21.25	20.03	1.203	37.8	85.1			
2	+3 "	40	Serum	1.3459	0.960		7.36	7.36	20.72	19.57	1.152	35.0	127.9	0.804	0.815	1 013
			Cells			0.639			16.84	15.73	1.114	35.0	104.2			
3	4 "	40	Serum	1.3459	0.960		7.34	7.31	21.38	20.06	1.324	40.2	121.7	0.776	0.821	1.057
			Cells			0.651			16.83	15.55	1.280	40.2	99.9			
4	7 "	40	Serum	1.3467	0.957		7.34	7.34	21.59	20.34	1.253	38.0	115.8	0.808	0.872	1.078
			Cells			0.653			17.64	16.43	1.209	38.0	101.0			
5	15 "	40	Serum	1.3468	0.955		7.31	7.36	20.89	19.73	1.161	35.2	116.0	0.815	0.845	1.036
			Cells			0.659			17.20	16.08	1.120	35.2	98.1			
6	4 hrs.	40	Serum	1.3491	0.943		7.36	7.43	19.34	18.42	0.924	28.0	117.6	0.782	0.822	1.050
			Cells			0.659			15.28	14.39	0.892	28.0	96.7			

positive test for sugar by the Benedict qualitative method and positive tests for albumin by the heat-acetic acid test were found in the late samples.

The excretion of both bromide and chloride was stimulated by the injection of bromide and the excretion of chloride was stimulated by the injection of chloride. The results are shown in Tables II, IV, VI, and VIII. Several interesting points are to be noted.

1. The concentration of chloride in the urine was only partly proportional to the concentration in the serum. For some time

TABLE IV

Experiment XXIV.   Sodium Chloride Injection.   Analysis of Urine

Total chloride excretion = 18 mM, or 18 per cent of amount injected (on the assumption that the rate of excretion is 0.08 mM per minute in Sample 8 and 0.01 mM per minute in Sample 10).

Sample No.	Time	Amount	pH, colorimetric	Total $[CO_2]$	[Cl]	Total Cl	Urine per min.	Cl per min.
	min.	*cc.*		*mM per l.*	*mM per l.*	*mM*	*cc.*	*mM*
1	Control		7.15	27.02	90			
2	"	1.5	7.09	41.63				
3	5	8.0	6.78	11.88	186	1.5	1.6	0.30
4	12	11.0	7.02	13.24	213	2.3	1.6	0.34
5	19	13.0	6.90	11.30	208	2.7	1.6	0.37
6	25	8.5	6.85	10.76	204	1.7	1.4	0.29
7	36	9.0	6.85	11.80	192	1.7	0.8	0.15
8	110		6.90	12.64				
9	160	14.8	6.90	25.41	87	1.3	0.3	0.03
10	250		7.47	45.60				

after the injection of bromide, the chloride excretion in mM per minute was considerably increased in spite of the fact that the serum chloride was lowered. In fact, in one sample of Experiment XXI, Tables V and VI, there was a pronounced chloride excretion when the serum value was below Ambard and Weill's (8) threshold value of 96.3 mM of chloride per liter. However, although the excretion of chloride per minute was increased in these cases, the chloride concentration in the urine was diminished in some of the samples.

Just as it seemed logical to assume in the preceding paper (1)

ay 23, 128; dog weigh   hou.

Sample No.	Time	Amount of blood (cc.)		Index of refraction	Hematocrit	H₂O (gm. per cc.)	H₂O (gm. per gm.)	pH, colorimetric	[Br] (m.m per kg. H₂O)	[Cl] (m.m per kg. H₂O)	$\frac{[Br]}{[Cl]}$	$\frac{[Br]_c}{[Br]_s}$	$\frac{[Cl]_c}{[Cl]_s}$	$\frac{r_{Cl}}{r_{Br}}$	$\frac{[Br+Cl]_c}{[Br+Cl]_s}$
1	−13 min.	40	Serum	1.3481		0.940		7.35		122.9			0.787		0.787
			Cells		0.532		0.669			96.8					
2	+3 "	80	Serum	1.3426		0.966		7.36	112.9	94.2	1.199	0.882	0.761	0.864	0.827
			Cells		0.297		0.629		99.6	71.7	1.389				
3	15 "	60	Serum	1.3455		0.952		7.32	68.4	97.9	0.699	0.937	0.730	0.779	0.815
			Cells		0.444		0.638		64.1	71.5	0.896				
4	50 "	40	Serum	1.3469		0.945		7.29	64.9	96.8	0.671	0.943	0.732	0.777	0.817
			Cells		0.494		0.651		61.2	70.8	0.865				

that respiratory activity may not be solely dependent on the concentration of a single ion or substance in the blood, so the evidence here points to the possibility that the excretory activity for a certain ion may not be solely dependent on the concentration of that single ion in the blood.

2. It is also seen in Tables VII and VIII that qualitatively the changes in the concentration of chloride in the urine were accompanied by changes in the concentration of bromide of a magnitude such that the ratio $\dfrac{[Br]_u}{[Cl]_u}$ was not far different from the ratio

TABLE VI

Experiment XXI. Sodium Bromide Injection. Analysis of Urine

Total bromide excretion = 37.1 mм, or 16 per cent of amount injected.
Total chloride excretion = 64.9 mм, or 28 per cent of amount injected.

Sample No.	Time	Amount	Index of refraction	Specific gravity	[Br]	[Cl]	$\dfrac{[Br]_u}{[Cl]_u}$	Total Br	Urine per min.	Br per min.	Cl per min.
	min.	*cc.*			*mM per l.*	*mM per l.*		*mM*	*cc.*	*mM*	*mM*
1	Control	53	1.3466	1.038		303.0					
2	6	38	1.3389	1.021	107.0	142.0	0.75	4.1	6.33	0.68	0.90
3	15	85	1.3363	1.016	92.6	137.8	0.68	7.9	9.45	0.88	1.30
4	30	130	1.3366	1.016	85.0	148.8	0.57	11.1	8.67	0.74	1.28
5	80	60	1.3387	1.023	107.0	210.4	0.51	6.6	1.20	0.13	0.25
6	220	35	1.3442	1.038	127.2	245.0	0.52	4.6	0.25	0.03	0.06
7	380	22	1.3495	1.055	87.2	211.0	0.42	1.9	0.14	0.01	0.03
8	510	13	1.3511	1.056	69.3	179.0	0.49	0.9	0.10	0.01	0.02

$\dfrac{[Br]_s}{[Cl]_s}$. Nevertheless, the former was consistently lower than the latter. This suggests that there is some tendency on the part of the kidneys to retain bromide and excrete chloride.

3. In Experiment XXIII the total chloride excretion caused by the sodium chloride injection was equal to 35 per cent of the amount injected. Referring to the bromide injection experiments, it is seen that the chloride excretion in Experiments XXI and XXII was 28 per cent and 35 per cent respectively of the total halide injection. In all three of these experiments the dosage was 10 cc. of 2.0 N salt solution per kilo of body weight, either sodium chloride or sodium bromide as the case might be. This indicates

TABLE VII

Experiment XXII. Sodium Bromide Injection

May 30, 1928; dog weight, 15.8 kilos; 3.8 gm. of barbital; 158 cc. of 2.0 N sodium bromide; death, 11 hours after injection.

Sample No.	Time	Amount of blood		Index of refraction	Hemato-crit	H_2O		pH, colori-metre	$[Br]$	$[Cl]$	$\frac{[Br]}{[Cl]}$	$\frac{[Br]_c}{[Br]_s}$	$\frac{[Cl]_c}{[Cl]_s}$	$\frac{r_{Cl}}{r_{Br}}$	$\frac{[Br+Cl]_c}{[Br+Cl]_s}$
		cc.				*gm. per cc.*	*gm. per gm.*		*mм per kg. H_2O*	*mм per kg. H_2O*					
1	−15 min.	40	Serum	1.3500		0.934		7.40		123.6					
			Cells		0.484		0.661			87.4			0.707		0.707
2	+3 "	75	Serum	1.3436		0.962		7.36	97.7	97.7	1.000				
			Cells		0.289		0.614		77.9	65.9	1.181	0.799	0.675	0.845	0.736
3	15 "	55	Serum	1.3469		0.947		7.30	68.7	103.6	0.663				
			Cells		0.388		0.627		58.1	76.9	0.755	0.846	0.742	0.878	0.784
4	4 hrs.	40	Serum	1.3502		0.934		7.19	64.5	96.5	0.668				
			Cells		0.515		0.654		58.2	72.2	0.807	0.902	0.748	0.830	0.810
5	8 "	40	Serum	1.3499		0.935		7.16	62.8	99.8	0.629				
			Cells		0.550		0.657		63.9	71.4	0.895	1.016	0.715	0.704	0.832

TABLE VIII

Experiment XXII. Sodium Bromide Injection. Analysis of Urine

Total bromide excretion = 56.7 mM, or 18 per cent of amount injected. Total chloride excretion = 109.5 mM, or 35 per cent of amount injected.

Sample No.	Time	Amount	Index of refraction	Specific gravity	Sugar	Albumin	[Br]	[Cl]	$\frac{[Br]_u}{[Cl]_u}$	Total Br	Urine per min.	Br per min.	Cl per min.
	min.	cc.					mM per l.	mM per l.		mM	cc.	mM	mM
1	Control	10	1.3523	1.040	Negative	?		194.0					
2	5	14	1.3434	1.028	"	Trace	81.7	166.8	0.49	1.1	2.80	0.23	0.47
3	10	49	1.3370	1.015	"	"	85.0	141.5	0.60	4.2	9.80	0.83	1.39
4	15	43	1.3370	1.014	"	"	81.6	149.0	0.55	3.5	8.60	0.71	1.28
5	30	160	1.3367	1.014	+	"	80.3	151.0	0.53	12.8	10.66	0 85	1.59
6	60	150	1.3372	1.016	Negative	"	88.3	168.2	0.52	13.2	5.00	0 44	0.84
7	125	113	1.3384	1.022	"	+	99.6	202.0	0.49	11.3	1.74	0.17	0.34
8	245	86	1.3396	1.024	"	+	99 6	207.0	0.48	8.6	0.72	0.07	0.15
9	665	29	1.3452	1.039	"	+++	70.3	153.4	0.46	2 0	0.07	0.01	0.01

that an injection of bromide caused practically as great a chloride excretion as an equal injection of chloride. Experiments XXIV and XXV cannot be directly compared with the bromide experiments because the dosage of injected chloride was smaller and the time of collection was shorter. Qualitatively they substantiate the conclusion stated above. (The total chloride excretion was 18 and 22 per cent respectively of the amount injected.)

Effect of Injection of Sodium Chloride and Sodium Bromide on Respiration and Blood Pressure

No blood pressure or respiratory tracings were made for the sodium bromide injections, but it was noticed that a moderate hyperpnea occurred some time after the start of the injection. In the case of two of the sodium chloride injections, tracings were made showing a rise of blood pressure and a hyperpnea following the injection. The rise of blood pressure did not begin for over 60 seconds after the start of the injection and was only 5 to 10 mm. of Hg. The hyperpnea was moderate in one case and quite vigorous in another. It began from 30 to 50 seconds after the start of the injection. This interval is longer than that preceding the hyperpnea of hydrochloric acid injection. This may be because a larger amount of the sodium chloride must be injected before a sufficient change in the acid-base balance is produced. Possibly the effect is due to the osmotic pressure changes produced by the hypertonic solution.

CONCLUSIONS

1. Red blood cells seem to have a stronger attraction for bromide ions than for chloride ions. The distribution ratios of bromide concentration between cells and serum are a uniform percentage greater than the chloride ratios. The presence of bromide lowers the chloride ratios even more.

2. The kidneys seem to have a greater ability to excrete chloride than bromide. The excretion of chloride following a sodium chloride injection is practically equal to that following a similar injection of sodium bromide. The excretion of chloride and bromide is not altogether proportional to the concentration of these ions in the serum.

3. Several less important observations were made. After the

injection of either sodium chloride or sodium bromide, the pH remains rather constant, but there are marked changes in the blood-water balance. The cells give up water, the water content of serum increases, the refractive index decreases, and the hematocrit reading falls. Most of these factors return toward normal and the hematocrit reading goes far above normal in the last few samples. Total carbon dioxide determinations were made only in the chloride injection experiments and showed a moderate drop in the value obtained after injection. The injection of either salt produces a moderate hyperpnea.

BIBLIOGRAPHY

1. Harkins, H. N., and Hastings, A. B., *J. Biol. Chem.*, **90**, 565 (1931).
2. Hastings, A. B., and van Dyke, H. B., *J. Biol. Chem.*, **92**, 13 (1931).
3. van Dyke, H. B., and Hastings, A. B., *J. Biol. Chem.*, **92**, 27 (1931).
4. Odaira, T., *Tohoku J. Exp. Med.*, **4**, 523 (1924).
5. Hanzlik, P. J., De Eds, F., and Tainter, M. L., *J. Pharmacol. and Exp. Therap.*, **21**, 217 (1923).
6. Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923–24).
7. Van Slyke, D. D., Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **65**, 701 (1925).
8. Ambard, L., and Weill, A., *Semaine méd.*, **32**, 217 (1912).

THE ACIDIC PROPERTY OF SUGARS

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Work reported in an earlier paper (Shaffer and Friedemann, 1930) on the formation of lactic and other saccharinic acids from reducing sugars led one of us some years ago to the hypothesis that the "activation" of reducing sugars which takes place in alkaline solution is the consequence of salt formation of the sugars, not only of one but of two or more acidic groups. (Similar views had been advanced much earlier by Nef and by Mathews.) Although it is well known that sugars behave as weak acids, there was until recently no valid evidence to justify the assumption of more than one acidic group. In order to test the hypothesis that sugars possess not only one, but several acidic groups and act as di- or polybasic acids, we undertook, about 3 years ago, the task of determining, by means of hydrogen electrode measurements, the amounts of base bound by glucose, fructose, and sucrose in rather concentrated NaOH solutions. High alkalinity was necessary to call forth to a detectable extent the postulated second or third acidic groups. Determinations were made also in moderately dilute hydroxide within the range used by previous workers on this subject, at which less than 1 equivalent of base is bound. From the data in the lower range of alkalinity the first dissociation constants have been again calculated.

In concentrated hydroxide solutions the difficulties, both experimental and theoretical, are considerable. The hydrogen electrode behaves rather poorly under such extreme conditions, while the strong reducing property of alkaline sugar solutions and the gradual *formation* of the relatively much stronger saccharinic acids from sugar decomposition were regarded as possible sources of

error.   Besides these, the liquid junction potential between NaOH (or NaOH + sugar) and KCl of bridge or calomel half-cell is of considerable and somewhat uncertain magnitude.   After a good deal of work these difficulties were, to a large extent, overcome, and results obtained which appear to leave no doubt that glucose, fructose, and sucrose possess at least two and, at high alkalinity possibly three acidic groups.   At least two, and possibly three different sugar ions, quite apart from decomposition or "dissociation" (Nef), may, therefore, be present in alkaline sugar solutions, the relative concentration of each ion depending of course on pH.

In a paper published in May, 1929, but which first came to our attention after a preliminary report[1] of our work had been presented, Hirsch and Schlags (1929) showed the dibasic character of a number of sugars.   The data here reported may, therefore, be regarded as confirmation and extension of the results of these authors.   (In their paper the earlier work on the acidic property of sugars is reviewed.)   Hirsch and Schlags based their calculations of the amount of base bound on measurements of electrical conductivity of NaOH solutions in the presence and absence of added sugar, the pH of the solutions containing sugar being determined, as in our work, by the hydrogen electrode.   The highest concentration of NaOH and of the several sugars used by them was 0.25 M.   They calculated both first and second (apparent) dissociation constants, the latter on the assumption that the sugars react (in the solutions used) only as dibasic acids.   Their values for glucose, fructose, and sucrose are shown for comparison with our own in Table VI.   The fact that we obtain, in general, similar values by a different method gives weight to the results of both.

Theory of the Calculation of Base Bound

The addition of sugar to an aqueous alkali solution causes a shift (lowering) of the pH, due to salt formation between sugar acid and base.   This shift is reflected by a change in E.M.F. of the cell,

$$\text{Pt} \mid \text{H}_2, \text{NaOH}_c \mid \text{KCl (saturated), HgCl} \mid \text{Hg}$$
$$\quad (a) \cdot \qquad\qquad (\pi) \qquad\qquad\qquad\qquad (b)$$

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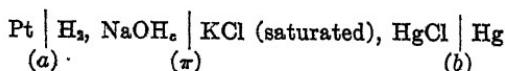
[1] Reported at the meeting of the American Chemical Society at Minneapolis, September, 1929.

and conversely, from the change in E.M.F. the amount of sugar salt formed may be calculated.   Given the two cells

$$\text{Pt}\quad \underset{(a_1)}{\text{H}_2,\ \text{NaOH}_c} \ \bigg|\ \underset{(\pi_1)}{\text{KCl (saturated)},\ \text{HgCl}} \ \bigg|\ \underset{(b)}{\text{Hg}}$$

$$\text{Pt}\ \bigg|\ \underset{(a_2)}{\text{H}_2,\ \text{NaOH}_c + \text{sugar}} \ \bigg|\ \underset{(\pi_2)}{\text{KCl (saturated)},\ \text{HgCl}} \ \bigg|\ \underset{(b)}{\text{Hg}}$$
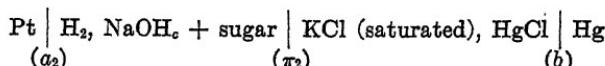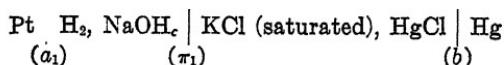
in which the initial NaOH concentration is the same in both, it follows that

$$(1)\qquad\qquad E_1 = \frac{RT}{F} \ln \frac{1}{(\text{H}^+)_1} + E_{(b)} + \pi_1$$

$$(2)\qquad\qquad E_2 = \frac{RT}{F} \ln \frac{1}{(\text{H}^+)_2} + E_{(b)} + \pi_2$$

where $\log \dfrac{1}{(\text{H}^+)_1}$ and $\log \dfrac{1}{(\text{H}^+)_2}$ represent the pH of the NaOH and NaOH + sugar solutions respectively, $E_{(b)}$, the potential of the calomel half-cell, and π, the liquid junction potentials.

By subtracting Equation 2 from Equation 1, we obtain the shift of E.M.F. (except for the *difference* in liquid junction potential π in the two cells considered below).

$$(3)\qquad\qquad E_1 - E_2 = \frac{RT}{F} \ln \frac{(\text{H}^+)_2}{(\text{H}^+)_1}$$

Substituting for $(\text{H}^+)_1$ and $(\text{H}^+)_2$ the corresponding values of $\dfrac{K_a \cdot (\text{H}_2\text{O})}{(\text{OH}^-)}$ gives:

$$(4)\qquad\qquad E_1 - E_2 = \frac{RT}{F} \ln \frac{(\text{OH}^-)_1 \cdot K_a(\text{H}_2\text{O})_2}{(\text{OH}^-)_2 \cdot K_a(\text{H}_2\text{O})_1}$$

$$(5)\qquad\qquad E_1 - E_2 = \frac{RT}{F} \ln \frac{(\text{OH}^-)_1}{(\text{OH}^-)_2} + \frac{RT}{F} \ln \frac{K_a(\text{H}_2\text{O})_2}{K_a(\text{H}_2\ \text{O}}$$

The value of the last member of Equation 5 may be disregarded in our experiments by the assumption that the activity of water in

an NaOH solution is only slightly changed by the formation of sugar salt.   Equation 5 may then be written,

$$(6) \qquad E_1 - E_2 = \frac{RT}{F} \ln \frac{C_1\,\gamma_1}{C_2\,\gamma_2}$$

where γ_1 and γ_2 are the activity coefficients, and C_1 and C_2 the concentrations of NaOH, without and with the sugar, respectively.

Since the ionic strength of the solution remains about the same after the addition of sugar, we may further assume the two activity coefficients for each pair of solutions to have the same value, and thus obtain the simplified equation (applicable only to each *pair* of solutions, of the same initial NaOH concentration, one with and one without sugar)

$$(7) \qquad E_1 - E_2 = \frac{RT}{F} \ln \frac{C_1}{C_2} = 2.303\,\frac{RT}{F} \log \frac{C_1}{C_2}$$

which for 25° becomes

$$(8) \qquad \frac{E_1 - E_2}{0.05912} = \log \frac{C_1}{C_2}$$

By means of Equation 8, the concentration of free NaOH in the presence of the sugar C_2, can be calculated from the "shift" in E.M.F. (C_1, the concentration in absence of sugar being known by titration), provided that the shift is first corrected for the *difference* of liquid junction potential at (π) between Equations 1 and 2.

For calculation of the liquid junction potentials, we have used the Henderson equation, in spite of the fact that it is theoretically applicable only to dilute solutions.   The modified equation of Cumming (1912), developed for higher concentrations, was less readily applicable because the necessary transport numbers were not available for 25°.

This equation was tested experimentally by one of us (U.) by measurements of the three cells

Pt H_2, NaOH$_c$ | saturated KCl, HgCl | Hg

Pt H_2, NaOH$_c$ | 0.1 M KCl, HgCl | Hg

Hg | HgCl, saturated KCl | 0.1 M KCl, HgCl | Hg
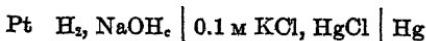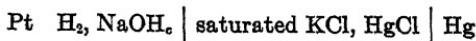
and by measuring also another set differing only by the addition of glucose to the NaOH solutions. The results were regarded as justifying application of the equation to the range of concentration of our solutions. Use of Cumming's equation would have given slightly greater values, and a small increase in values for the "shift." The correct fractions of base bound may, therefore, be slightly larger than the values calculated; but the differences between results by the two equations are probably within the experimental error.

In the calculation of liquid junction potential, which is simple with pure NaOH solutions, difficulty is encountered when the equation is applied to the alkali-sugar solutions in which the concentrations of free NaOH and of sugar-salt are not exactly known. The procedure followed was first to use in Henderson's equation the approximate concentration of free NaOH obtained from Equation 8. The resulting junction potential was then used to correct the value of the shift $(E_1 - E_2)$, and free NaOH again computed. This new value when substituted in the Henderson equation yielded a new junction potential value which, in most cases, was close to the first. When necessary the cycle was repeated. As a rule the third repetition yielded results within 0.1 mv. of the second. The sign of the liquid junction potential is such that for NaOH alone it is to be added to the observed E.M.F. (except at the two lowest NaOH concentrations). Being due chiefly to the hydroxyl ion, it is lowered by the presence of the sugar; hence, the true shift is greater than the observed difference of potential, $E_1 - E_2$, by the *difference* between the liquid junction potential in the presence and absence of sugar (by from 0.4 to 7.0 mv.). When correction for liquid junction potential is omitted, although the fraction of base bound per mol of sugar is somewhat decreased, the dibasic character of the sugars is still clearly shown.

The shift, $E_1 - E_2$, corrected for *difference* in liquid junction potential, divided by 0.05912, gives by Equation 8 the logarithm of $\dfrac{C_1}{C_2}$, the ratio (R) of free NaOH concentrations without and with sugar, the concentration in absence of sugar being known by titration. Then

(9) $(NaOH)_{c_1} \div R = (NaOH)_{c_2}$, and

(10) $(NaOH)_{c_1} - (NaOH)_{c_2} = (Na)$ salt

the amount of base bound by sugar as salt. Dividing this value by the molar concentration of sugar used gives the molar equivalents of base neutralized per mol of sugar at the observed pH. These quantities are recorded in Tables II to V under "base fraction."

The pH of the NaOH + sugar solutions was determined as usual from E_h (corrected for liquid junction potential). The value of the calomel cell (saturated KCl) used throughout as working reference electrode was based upon its potential when combined with the hydrogen electrode in 0.05 M acid potassium phthalate, assuming its pH to be 3.97 (Clark, 1928) and ignoring the liquid junction potential between phthalate solution and saturated KCl-agar bridge. Corrections were made in all cases for H_2 pressure.

The apparent dissociation constants were calculated as follows: The constants are "apparent" in that they include the activity coefficient of the salt. Change of the "apparent" constants with ionic strength of the solution is ignored. The values of pK_1' (the first constant) were obtained by applying the Henderson-Hasselbalch equation

$$(11) \qquad pK_1' = pH - \log \frac{[salt]}{[HA]}$$

to the data of experiments within the pH range at which less than 0.5 base equivalent is bound, and the average values taken. This equation is valid only at pH regions where it may be assumed that the amount of base bound by the second acidic group is negligible. Its use has the advantage of giving values for pK_1 independent of the increasing errors at higher pH range.

The second constants (pK_2') were calculated from the expression

$$(12) \qquad 2a - pK_1' = pK_2'$$

in which a is the pH at which the acid (sugar) binds 1 mol-equivalent of base. This equation is the same as Hirsch and Schlags' (1929), $\frac{1}{2}(M_1 + M_2) = a$, and is derived as follows: Multiplying together the equations for the first and second dissociations of a dibasic acid,

$$(13) \qquad \left(K_1 = \frac{(H^+)\,(HA^-)}{(H_2A)} \right) \times \left(K_2 = \frac{(H^+)\,(A^=)}{(HA^-)} \right) =$$

$$\frac{K_1 \times K_2}{(H^+)^2} = \frac{(A^=)}{(H_2A)} \right)$$

substituting B_2A (dibasic salt concentration) for A^-, K' for K, and taking the logarithm of both sides, gives

$$(14) \qquad 2\ pH - pK_1' - pK_2' = \log \frac{(B_2A)}{(H_2A)}$$

Assuming the total sugar to be present as H_2A, BHA, and B_2A, the total *base* of (BHA) $+$ (B_2A) equals the total *sugar* of H_2A $+$ HBA $+$ B_2A; *i.e.*, the sugar binds 1 equivalent of base, when (Ba_2A) $=$ (H_2A). Let the pH at which this is true be a, then $2a - pK_1' = pK_2'$. The values of a were determined graphically from curves constructed from the data (see Figs. 1 to 5).

Experimental Details—The NaOH solutions were prepared free from CO_2 by the method of Sörensen (1909), and standardized by titration against standard HCl and acid potassium phthalate solutions. The sugars used were of high purity. The glucose was from Eastman; fructose was obtained through the courtesy of Dr. Hudson, from the United States Bureau of Standards. Sucrose was purified from commercial cane sugar by decolorization with norit, crystallization by addition of 4 volumes of 95 per cent alcohol, and drying *in vacuo*.

The final NaOH concentrations ranged from 0.012 to 1.197 M, and the sugar concentrations from 0.1 to 0.4 and 0.6 M. The cell measurements were made in a constant temperature bath, usually at 25° ± 0.05°. The hydrogen was commercial tank electrolytic gas, purified by passage over platinized asbestos, in a quartz tube, heated by an electric furnace to a dull red, then bubbled first through water, then through a sample of the alkali solution being analyzed (in the bath), and finally into the hydrogen electrode vessel.

Saturated KCl-calomel electrodes and bridges of saturated KCl in 1.5 per cent agar were used. The potentiometer was type K of Leeds and Northrup, with a calibrated Weston cell. Two different samples of each NaOH solution, alone and with each sugar concentration, were prepared and read separately. Potentiometer readings were made approximately 10 minutes after the bridge was in position, or 15 to 20 minutes after mixing the solutions. Duplicate determinations often agreed within 0.1 mv. When less satisfactory, often the case with the high NaOH concentrations, an

average was taken, or the experiment was repeated until steady and apparently reliable values were obtained.

Error due to saccharinic acid formation was minimized by making the measurements as rapidly as possible. Readings over a 2 hour period of 0.4 M glucose in 0.666 M NaOH at 25° showed a drift, detectable after 10 to 20 minutes, of about 0.1 mv. per 10 minutes. This indicates that at high alkalinity (or temperature) a considerable error may be introduced in the case of reducing sugars, if the potential readings are much delayed. Error due to this behavior possibly may account for some of the scattered results observed especially with glucose at high alkali concentration. It was necessary to replatinize the electrodes frequently when used in the alkali-sugar solutions; otherwise erratic results were often obtained.

Results

NaOH Solutions without Sugar—Table I gives the concentrations (titration values) of the NaOH solutions, the calculated liquid junction potentials, the corrected E_h values, and the corresponding pH numbers, in absence of sugar. These data not only represent the base-line for calculation of results obtained in the presence of the sugars, but indicate also the probable limits of error in the measurements and in the estimates of liquid junction potentials.

Multiplying the NaOH concentrations [NaOH] by the corresponding activity coefficients (determined by Harned (1925) at 25° in cells without liquid junction) gives hydroxyl ion activities, (OH^-). The product $(OH^-) \times (H^+)$ should then equal the ionic product of water, K_{H_2O}, or be slightly less than this value, to the extent that the activity of water in NaOH solutions is less than 1. Taking the logarithmic form of this relation

$$pOH + pH = pK_{H_2O} + \log \frac{1}{(H_2O)}$$

Column 8 of Table I gives the sum pOH + pH and Column 9 the deviation of this sum from 13.99, the "best" value for pK_{H_2O} at 25° (International Critical Tables, **6**, p. 152). Instead of the deviation approaching zero at the lowest NaOH concentration it does so around 0.4 to 0.5 M. drifting with a semblance of regularity on both sides of this region. The apparent approach to systematic

deviation suggests some theoretical basis, such as error in estimates of liquid junction potentials or in some of the constants. But we have not succeeded in reaching an acceptable explanation and shall,

TABLE I

NaOH Solutions. (No Sugar Present)

NaOH No.	NaOH	Liquid junction potential	E_h	pH	Activity coefficient γ NaOH (Harned)	pOH	pH + pOH	Deviation from 13.99
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
				25°				
	M	mv.						
1	0.0116	+0.7	−0.7037	11.903	0.91	1.977	13.88	−0.11
2	0.0233	+0.3	−0.7232	12.233	0.87	1.693	13.93	−0.06
3	0.0466	−0.4	−0.7408	12.530	0.825	1.416	13.95	−0.04
4	0.0698	−0.7	−0.7505	12.695	0.80	1.253	13 95	−0.04
5	0.0833	−0.9	−0.7537	12.750	0.79	1 182	13.93	−0.06
6	0.0933	−1.1	−0 7579	12.82	0.785	1.135	13.96	−0.03
7	0.111	−1.3	−0.7630	12.906	0.77	1 068	13 98	−0.01
8	0.166	−2.1	−0.7723	13.064	0.755	0.903	13.97	−0.02
9	0.222	−2.7	−0.7795	13.185	0.74	0.785	13.97	−0.02
10	0.333	−3.8	−0.7889	13.343	0.72	0.621	13.97	−0.02
11	0.444	−4.6	−0.7975	13.490	0.705	0.505	13.99	0
12	0.500	−5.1	−0.8002	13.535	0.70	0.457	13.99	0
13	0.555	−5.5	−0.8031	13.585	0.695	0.414	14.00	+0 01
14	0.666	−6.3	−0 8080	13.667	0.685	0.340	14.00	+0.01
15	0.777	−6.9	−0.8118	13.732	0.677	0.281	14.01	+0.02
16	0.888	−7.6	−0.8145	13.777	0 675	0.223	14.00	+0.01
17	1.197	−9.1	−0.8246	13.948	0 695	0.080	14.03	+0.04
				17°				
		For 25°						
20	0.095	− (1.1)	−0.7556	13.133				
21	0.190	− (2.3)	−0 7723	13.424				
22	0.285	− (3.3)	−0.7833	13.615				
23	0.380	− (4.1)	−0.7906	13.74				
24	0.570	− (5.6)	−0.7992	13.89				
25	0.720	− (6.6)	−0.8068	14.023				

therefore, merely accept the variations as indications of the probable errors. Eleven of the seventeen values are within 0.03 unit and fourteen within 0.04 unit of the theoretical, the average devia-

tion being +0.025 and −0.013. It seems likely that the probable error in pH determinations does not exceed ±0.028. This corresponds to ±1.66 mv. as the probable error in E_h, from which the amounts of bound base are calculated. In the lower range of alkalinity where the "shift" is large an error of this magnitude would be of small consequence, but at the highest range of alka-

TABLE II

Sucrose at 25°

NaOH No.	NaOH	Sucrose	Δ liquid junction potential	Shift corrected	Base fraction	pH	pK₁
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	M	*M*	*mv.*	*volt*			
1	0.0116	0.1	+0.3	0.0259	0.07	11.46	12.584
2	0.0233	0.1	+0.2	0.0243	0.14	11.82	12.608
3	0.0466	0.1	+0.9	0.0233	0.28	12.15	12.56
4	0.0698	0.1	+1.0	0.0213	0.39	12.345	12.54
5	0.0832	0.1	+1.0	0.0186	0.43	12.44	12.56
6	0.0931	0.1	+1.1	0.0189	0.49	12.51	12.53
8	0.166	0.1	+1.4	0.0158	0.76	12.80	(12.30)
10	0.3326	0.1	+1.5	0.0100	1.08	13.17	
12	0.500	0.1	+1.7	0.0089	1.47	13.39	
14	0.666	0.1	+1.7	0.0069	1.58	13.56	
1	0.0116	0.2	+0.4	0.0382	0.05	11.26	12.54
3	0.0466	0.2	+1.4	0.0367	0.18	11.90	12.56
6	0.0931	0.2	+1.7	0.0354	0.35	12.22	12.49
8	0.166	0.2	+2.1	0.0292	0.56	12.57	12.46
10	0.3326	0.2	+2.7	0.0209	0.92	13.00	(11.94)
12	0.500	0.2	+3.1	0.0180	1.26	13.24	
14	0.666	0.2	+3.2	0.0153	1.49	13.41	
5	0.0832	0.4	+1.8	0.0522	0.18	11.87	12.53
8	0.166	0.4	+2.9	0.0496	0.36	12.24	12.49
10	0.333	0.4	+4.3	0.0430	0.67	12.62	(12.30)

linity with low values for the "shift" it amounts to as much as 20 per cent or more. In the latter region the liquid junction potential correction also becomes large, which further limits the accuracy attainable by this method. These factors are probably responsible for the scattering of the data at highest alkalinity, to be observed by inspection of Figs. 1 to 5.

The data for sucrose, glucose, and fructose are shown in Tables

TABLE

Gl   se a

NaOH No.	NaOH	0.1 M glucose					0.2 M glucose					0.4 M glucose				
		Δ liquid junction potential	Shift corrected	Base fraction	pH	pK₁	Δ liquid junction potential	Shift corrected	Base fraction	pH	pK₁	Δ liquid junction potential	Shift corrected	Base fraction	pH	pK₁
	M	$mv.$	$volt$				$mv.$	$volt$				$mv.$	$volt$			
1	0.012	+0.4	0.0443	0.095	11.15	12.13	+0.5	0.0629	0.05	10.83	12.11	+0.5	0.0828	0.03	10.50	12.01
2	0.023	+0.7	0.0441	0.19	11.48	12.11	+0.8	0.0618	0.11	11.18	12.09	+0.8	0.0844	0.06	10.80	11.99
3	0.047	+1.2	0.0398	0.37	11.85	12.08	+1.4	0.0589	0.21	11.54	12.11	+1.5	0.0817	0.11	11.14	12.05
4	0.070	+1.3	0.0358	0.53	12.09	12.04	+1.6	0.0556	0.31	11.76	12.11	+1.8	0.0800	0.17	11.33	12.02
5	0.083	+1.4	0.0340	0.61	12.16											
6	0.093	+1.7	0.0332	0.68	12.26		+1.9	0.0519	0.41	11.95	12.11	+2.4	0.0720	0.26	11.68	12.13
7	0.111	+1.6	0.0304	0.77	12.39		+2.1	0.0501	0.48	12.06	12.09	+3.2	0.0704	0.39	11.87	12.06
8	0.166	+2.1					+2.6	0.0414	0.67	12.36		+3.7	0.0642	0.51	12.10	12.08
9	0.222	+2.0	0.0133	0.94	12.94		+2.8	0.0328	0.80	12.63		+5.1	0.0423	0.90	12.76	
10	0.333	+2.1	0.0114	1.20	13.14		+3.2	0.0275	1.10	12.86						
11	0.444	+1.9	0.0102	1.45	13.32											
12	0.500						+3.0	0.0151	1.11	13.23						
13	0.555	+1.5	0.0076	1.42	13.40		+3.1	0.0157	1.27	13.31		+4.5	0.0324	1.00	13.03	
14	0.666	+1.2	0.0055	1.29	13.56		+3.2	(0.0155)	1.51	13.40		+4.5	0.0247	1.03	13.25	
15	0.777	+3.5*	0.0163*	1.22*	13.45*							+4.3	0.0235	1.17	13.34	
16	0.888						+2.8	0.0113	1.58	13.57		+4.8	0.0210	1.24	13.41	
17	1.197	+7.0†	0.0287†	1.34†	13.46†							+4.9	0.0181	1.51	13.63	

* 0.3 M glucose
† 0.6 M glucose

TABLE IV

Glucose at 17°

NaOH No. (1)	NaOH (2)	Glucose (3)	Δ liquid junction potential (25°) (4)	Shift corrected (5)	Base fraction (6)	pH (7)	pK₁ (8)
	M	M	mv.	volt			
20	0.095	0 2	+1.7	0 0537	0.42	12.20	12.34
21	0.190	0.2	+2.6	0.0365	0.73	12.79	
22	0.285	0.2	+2.8	0.0273	0.95	13.14	
23	0.380	0.2	+2.9	0.0211	1.09	13.374	
24	0.570	0.2	+3.1	0.0144	1.25	13.64	

TABLE V

Fructose

NaOH No. (1)	NaOH (2)	Fructose (3)	Δ liquid junction potential (4)	Shift corrected (5)	Base fraction (6)	pH (7)
			25°			
	M	M	mv.	volt		
5	0.083	0.1	+1.6	0.0434	0.68	12.00
8	0.166	0.1	+2.1	0.0280	1.11	12.60
12	0.500	0.1	+2.4	0.0140	2 10	13.30
14	0.666	0 1	+2.5	0 0119	2.48	13.46
5	0.083	0.2	+1.7	0.0682	0 39	11.60
8	0.166	0.2	+3.0	0.0579	0.75	12.08
10	0.333	0.2	+3.6	0.0332	1.21	12.79
12	0.500	0.2	+3.7	0.0237	1.51	13.13
14	0.666	0.2	+3.5	0.0179	1.68	13.36
12	0.500	0.5	+5.0	0.0659	0.92	12.42
12	0.500	1.0	+7.0	0.1176	0.49	11.55
	0.008	0.02		0.0228	0.22	12.05
	0.030	0.02		0.0142	0.66	12.84
	0.083	0.02		0.0069	1.04	13.40
	0.166	0.02		0.0049	1.56	13.75
	0.008	0.04		0.0398	0.17	11.72
	0.030	0.04		0.0315	0.57	12.53

II to V. It should be noted in these tables that the figures in columns headed "Δ liquid junction potential" are *differences* between the calculated junction potentials in the presence and absence of sugar, the sign in all cases being such that the correction increases the observed $E_2 - E_1$. The figures in columns headed "shift corrected" include the liquid junction correction. From these values the corrected E_h for sugar solutions, from which pH was calculated, may be obtained (if desired for checking calculations) by subtracting the "shift" from the value of E_h for the corresponding NaOH solution given in Table I. The values for equivalents of base bound at different pH levels are plotted in Figs. 1 to 5.[2]
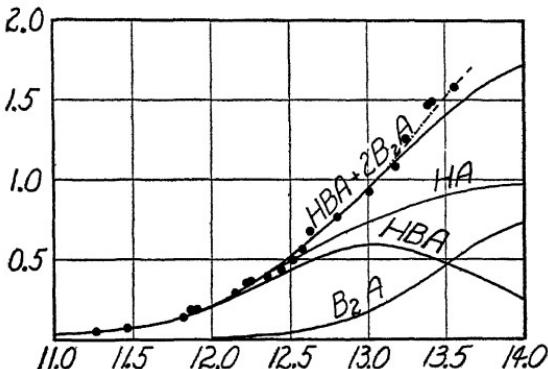
FIG. 1. Amount of base bound by sucrose at various pH levels, at 25°. The ordinate represents the base fraction; the abscissa, the pH.

Sucrose—The polybasic character of the sugars is most simply presented by comparing the amounts of base bound at given pH levels with the theoretical amounts bound by a monobasic acid having the same dissociation constant as that found for the first acidic group of the sugar. The data for sucrose will illustrate this. Values for pK_1' calculated from the results within the lower pH range, shown in Column 8 of Table II, average 12.54.

[2] One of us (U.) has since been able to confirm the values for equivalents of base bound at different pH levels by glucose, using a glass electrode and, thereby, eliminating liquid junction potentials. Results will be published later. Regarding the method see Urban, F., and Steiner, A., *J. Physic. Chem.*, **35**, 3058 (1931).

Curve HA in Fig. 1 represents the theoretical fraction present as salt (*i.e.*, the amount of base bound per mol) of a monobasic acid[3] having a dissociation constant, $pK' = 12.54$. It will be seen that this curve approximately fits the experimental points only below about 0.4 salt fraction. Above this sucrose binds more base, due to a second acidic group. The points locate a line which, instead of curving toward the horizontal, runs for a distance approximately straight. The slope of the straight line indicates the ratio of the first to second constants, as made clear by the theoretical treatment of the behavior of dibasic acids by Van Slyke (1922), Hirsch (1924), Michaelis (1922), and others.

If we similarly calculate the fractions of a dibasic acid present in the form of mono- and the dibasic salt[4] at different pH levels, the sum of $HBA + 2B_2A$ represents the total base bound by the two acidic groups. Curves are drawn on Fig. 1 to represent these fractions and the sum of base bound. It will be seen that the last named theoretical curve fits the somewhat scattered experimental points fairly well up to a total salt fraction (base equivalents bound) about 1. The values of the constants from which the curves shown are calculated, and which best fit these data, are pK_1' $= 12.60$, $a = 13.06$, and $pK_2' = 13.52$. The corresponding values reported by Hirsch and Schlags are 12.51, 13.02, and 13.52.

The points at highest pH indicate more base to be bound than corresponds to two acidic groups and hence suggest a third, which begins to function perceptibly above pH about 13. The upper dotted curve as drawn represents the theoretical total base bound if the third constant be taken as 14.20. This value roughly fits the data. There are, however, large errors in these results at high pH which make the value for pK_3' only a rough approximation. The errors in this region are not alone experimental but are due chiefly to the mathematical relation which at increasing pH exaggerates increasingly the effect of small experimental errors on

[3] Data for this curve are calculated from the equations $\log R = pH - pK'$, and $salt_m = \dfrac{R}{R+1}$, in which $R = \dfrac{[BA]}{[HA]}$ and $salt_m$ is the fraction of the acid present as salt.

[4] The mono- and dibasic salt fractions are calculated from the equation $\dfrac{HA^-}{S} = \dfrac{K_1 \cdot H^+}{K_1 H^+ + H^{+2} + K_1 \cdot K_2}$ in which S is the sum, $H_2A + HA^- + A^-$.

the calculated results. For this reason the accuracy attainable by this method of determining dissociation constants declines greatly with the magnitude of the constants. The existence of the third constant for sucrose is based on four points with "shifts" of 6.9 to 18 mv. of which 1.7 to 3.2 is the difference in liquid junction potential. If the shifts were about 2 mv. less the points would fall near the line for a dibasic acid. There is, however, no reason to suppose that the values for the shift are too high, and we, therefore, tentatively conclude that a third acidic group exists, the constant for which is of the order given.

Glucose—From what has been said above concerning sucrose, the significance of Figs. 2 and 3 for glucose will perhaps be evident by inspection. The experiments at 25° are quite consistent in the
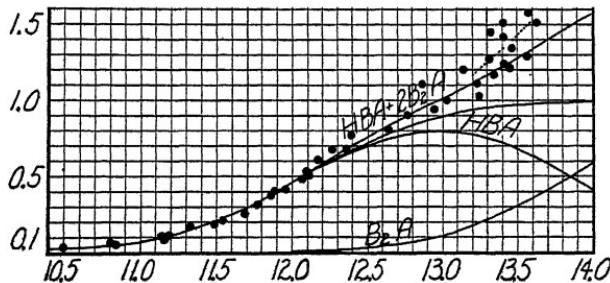
Fig. 2. Amount of base bound by glucose at various pH levels, at 25°. The ordinate represents the base fraction; the abscissa, the pH.

lower pH range, and yield values for pK_1' shown in Table III, the best value being 12.09. Considering the wide range of sugar as well as alkali concentrations used, the results seem quite satisfactory. The amounts of base bound above pH about 12.1 give evidence again of a straight line instead of the curve required for a monobasic acid. The value for a is 12.97 and for pK_2', 13.85. The corresponding values of Hirsch and Schlags are pK_1, 12.11 ($a = 12.96$) and pK_2, 13.81. Curves showing the theoretical mono- and dibasic salt fractions corresponding to our constants, and the sum of base in both, are drawn on Fig. 2. It will be seen that although the experimental points scatter considerably in the upper range, the theoretical curve for total base appears to represent the mean. Above pH about 13 there appears to be evidence

of a third acidic group, though the points are too scattered to permit satisfactory estimate of the value of its constant. For the present we merely suggest its existence.

Fig. 3 shows a few results with glucose at 17°. The values of the constants which appear to fit the data best are pK$_1$', 12.34; pK$_2$', 14.10. It is of interest to note that the change of the constants with temperature between 25° and 17° is not very different from the change of pK$_{H_2O}$. (pK$_1$', 12.34 − 12.09 = 0.25; pK$_{H_2O}$, 14.26 − 13.99 = 0.27.)

Fructose—The results for fructose are rather less consistent than those for sucrose and glucose perhaps due in part to its more rapid decomposition in alkaline solution. The data for 25° are plotted
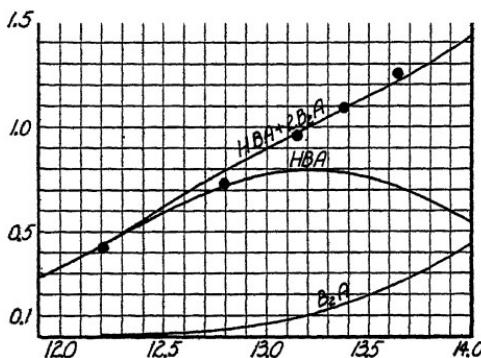
FIG. 3. Amount of base bound by glucose at various pH levels, at 17°. The ordinate represents the base fraction; the abscissa, the pH.

in Fig. 4. The values of the constants which seem best to fit the data are for 25°, pK$_1$', 11.68; pK'$_2$, 13.24. The first is close to Hirsch and Schlags value, 11.69, while their values for pK$_2$' is 13.81, the same as found by them for the second constant of glucose. In view of the close structural relations it is attractive to suppose that the second acidic groups may be the same in both sugars and may have the same constants. But our data do not permit selection of pH 12.75 as the a point, which is necessary to give the Hirsch and Schlag value for pK$_2$'. The discrepancy in the two values for pK$_2$' will require further work to explain.

Again with fructose the points at the highest pH indicate overlapping of a third acidic group, though because of the scattering of

the points and the reasons already mentioned, it is not possible to locate the line definitely nor to estimate the value of the hypothetical constant. In the hope of getting more reliable data with fructose at high alkalinity, a series of measurements were made at
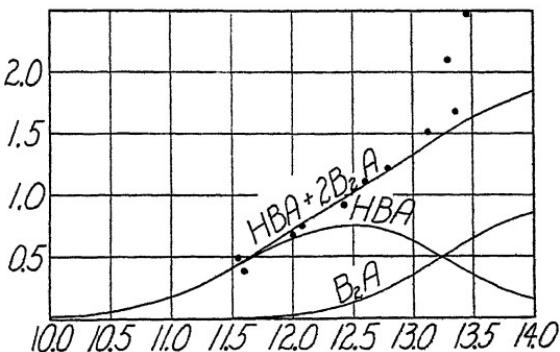
Fig. 4. Amount of base bound by fructose at various pH levels, at 25°
The ordinate represents the base fraction; the abscissa, the pH.
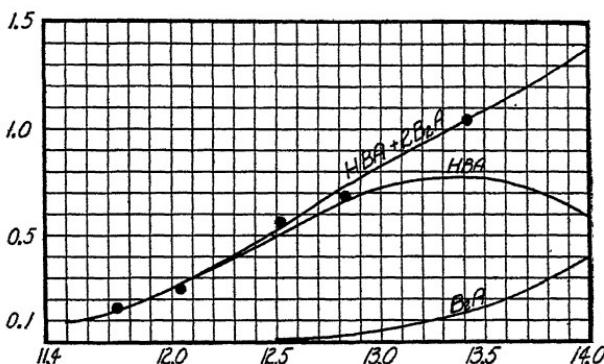
Fig. 5. Amount of base bound by fructose at various pH levels, at 4°.
The ordinate represents the base fraction; the abscissa, the pH.

4° with lower sugar and NaOH concentrations. The results are shown in Fig. 5. The points are not very consistent. The best values for the constants appear to be, pK_1', 12.50; a, 13.34; pK_2', 14.18. In 0.166 N NaOH and 0.02 M fructose there is again evidence of a third acidic group, though the shift is so small (4.9 mv.)

that allowing a small error would so decrease the base bound as to make assumption of the third group unnecessary. The constants obtained for the three sugars together with the values of Hirsch and Schlag are brought together in Table VI.

TABLE VI

Apparent Dissociation Constants

		pK_1'	pK_2'	(a)	$K \times 10^{13}$		Ratio $\dfrac{K_1'}{K_2'}$
					K_1'	K_2'	
Glucose							
25°	Hirsch-Schlags	12.107	13.813	12.96	7.8	0.154	51
25°	Authors	12.09	13.85	12.97	8.1	0.14	58
17°	"	(12.34)*	(14.10)*	13.22	(4.6)*	(0.08)*	57
Fructose							
25°	Hirsch-Schlags	11.693	13.807	12.75	20.3	0.156	130
25°	Authors	11.68	13.24	12.46	20.9	0.58	36
4°	"	12.50	14.18	13 34	3.2	0.066	48
Sucrose							
25°	Hirsch-Schlags	12.513	13.523	13.018	3.1	0.30	10
25°	Authors	12.60	13.52	13.06	2.5	0.30	8.3

* Based on a single determination of pK_1.

SUMMARY

The amounts of $NaOH$ neutralized by glucose (25° and 17°), by fructose (25° and 4°), and by sucrose (25°) were determined by means of the hydrogen electrode. All three sugars behave as dibasic acids. The following values were obtained for the dissociation constants at 25°: glucose, pK_1' 12.09, pK_2' 13.85; fructose, pK_1' 11.68, pK_2' 13.24; sucrose, pK_1' 12.60, pK_2' 13.52.

The data appear to indicate that with each of these sugars a third acidic group begins to function at high alkalinity; but because of large errors in this region the existence of the third group must be regarded as uncertain.

BIBLIOGRAPHY

Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition (1928).

Cumming, A. C., *Tr. Faraday Soc.*, **8**, 86 (1912).

Harned, H. S., *J. Am. Chem. Soc.*, **47**, 676 (1925).

Hirsch, P., *Biochem. Z.*, **147**, 433 (1924).

Hirsch, P., and Schlags, R., *Z. physik. Chem.*, **141,** 387 (1929).

Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 23 (1922).

Shaffer, P. A., and Friedemann, T. E., *J. Biol. Chem.*, **86,** 345 (1930).

Sörensen, S. P. L., *Biochem. Z.*, **21,** 168 (1909).

Van Slyke, D. D., *J. Biol. Chem.*, **52,** 525 (1922).

THE DETERMINATION OF PYRUVIC ACID AND THE PREPARATION OF LITHIUM PYRUVATE

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In the course of studies with blood (1) and other tissues in which lactic acid oxidation was indicated, we were confronted with the necessity of determining pyruvic acid in small amounts. Dr. T. E. Friedemann drew our attention to the work of Lieben, in which pyruvic acid is reduced to lactic acid and thus determined. A method was developed based on this procedure. After the completion of the greater part of our studies, Kendall and Friedemann (2) published a paper dealing with this reaction, and, thus, our findings may be considered supplementary to those of the above workers.

Inasmuch as a salt of pyruvic acid useful for recovery studies has not been described, we sought to prepare one and obtained a lithium salt which apparently has the composition $LiC_3H_3O_3 \cdot H_2O$. With this salt as a standard, the method described gives recoveries of 98.5 per cent when corrected for 2 per cent loss inherent in the lactic acid method.

HISTORICAL

Since the discovery of pyruvic acid by Berzelius (3) a number of methods for its identification and determination have been reported. Useful color reactions are described by Simon (4), Quastel (5), Posternak (6), Alvarez (7), and Garzarolli-Thurnlackh (8). Anderson, Peterson, and Fred (9) state that a color reaction is obtained with Uffelman's reagent. The phenylhydrazone of pyruvic acid first described by Fischer (10) and Fischer and Jourdan (11) has been used in various ways for quantitative determinations by de Jong (12), MacLean (13), Simon and Piaux (14), and Hahn, Fischbach, and Haarmann (15). The p-nitrophenylhydrazone, a less soluble derivative, first described by Hyde (16), has been used extensively by Neuberg and coworkers. More recently 2,4-dinitrophenylhydrazine has

been used by Neuberg and Kobel (17). The employment of this for quantitative separation and estimation of acetaldehyde, methylglyoxal, and pyruvic acid is described by Simon and Neuberg (18). The semicarbazone and its Ca and Zn salts have been used in isolation studies by Kostytschew and Soldatenkov (19). Methods based on partial or complete oxidation by a variety of oxidizing agents are described by Beilstein and Wiegand (20), Quastel (5), Fernbach and Schoen (21), Holleman (22), Levene and Meyer (23), Bleyer and Braun (24), and Mazé and Ruot (25). Criticisms of some of these methods are offered by Denis (26), Evans and Witzemann (27), Lieben (28), and Hatcher and Hill (29). Wieland (30), using the reaction noted by Neuberg (31), describes the conditions under which NaOI quantitatively converts pyruvic acid to iodoform. (See Hatcher and Mueller (32) for critical studies.) Warburg, Kubowitz, and Christian (33) decarboxylate pyruvic acid with yeast carboxylase and determine the CO_2 manometrically. A biological method capable of detecting 0.001 mg. of pyruvic acid is described by Mendel, Bauch, and Strelitz (34). Cook (35) utilizes the bisulfite-combining capacity of pyruvic acid for quantitative determination.

The reduction of pyruvic acid to lactic acid was accomplished in 1863 by Wislicenus (36) and Debus (37), but it was not until 1923 that this reaction was employed for quantitative determination. Lieben (28) boiled pyruvic acid with Zn and HCl under a reflux for some hours and determined the resulting lactic acid by the Fürth-Charnass procedure with recovery of 96 to 109 per cent. Laufberger (38) and Krishna and Sreenivasaya (39) found the Lieben method unsuited to the determination of small amounts of pyruvic acid. The latter workers, by modifying the reduction procedure (employing Zn-Cu couple and H_2SO_4) and by using the Friedemann-Cotonio-Shaffer (40) method for lactic acid determination, obtained consistent recoveries of 80 per cent over a wide range of concentrations. Kendall and Friedemann (2) further modified the Lieben procedure and obtained recoveries of 94 per cent.

A number of salts of pyruvic acid including that of lithium were prepared by Berzelius (3), but to our knowledge none has been used in recovery studies.

Analytical Procedure

Reduction—A (25 to 150 cc.) sample of a dilute solution of pyruvic acid (0.5 to 75 mg.) is measured into a volumetric flask (50 to 500 cc.) and reduced by the addition of zinc dust (0.5 to 2 gm.), copper sulfate (2 to 4 drops of saturated solution), and H_2SO_4 (2 to 5 cc., 5 N). The flask is rotated vigorously for 1 minute, and intermittently for 4 minutes. When evolution of hydrogen has practically ceased the solution is diluted to volume and filtered. Aliquots are run for lactic acid. Theory, 1.0 cc. of 0.01 N I_2 = 0.44 mg. of pyruvic acid.

Lactic Acid Determination—The Friedemann-Cotonio-Shaffer method is used, modified as to acid reagent and apparatus (41). The oxidation is carried out by slow addition of 0.01 N KMnO$_4$ to the lactic acid solution to which has been added a mixture to give the following final composition: 0.1 N H$_2$SO$_4$, 0.2 M Na$_2$SO$_4$, and 1 per cent MnSO$_4$·4H$_2$O.[1]

Iodoform Reaction—An excess of 0.1 N iodine is run into a solution of pyruvic acid and diluted with water to approximately 100 cc. 5 cc. of 40 to 50 per cent NaOH are then introduced, mixed well, allowed to stand 5 minutes, acidified with 5 N H$_2$SO$_4$, and titrated with 0.1 N thiosulfate. The iodine consumption by reagents is corrected for by running blanks with an equal volume of iodine solution. Theory, 1.0 cc. of 0.1 N I$_2$ = 1.467 mg. of pyruvic acid.

Elementary Analysis—Lithium was determined on 0.5 to 1.0 gm. samples of lithium pyruvate by conversion to Li$_2$SO$_4$, carbon, and hydrogen by Pregl's micro technique.[2]

Pyruvic Acid Preparations

Purified pyruvic acid (64–67° at 13 mm. of Hg), obtained from the Eastman Kodak Company, was the starting material for all preparations.

Preparation A was the above material without further treatment.

Preparation B was Preparation A distilled at 1 to 2 mm. of Hg. The first fraction, up to 47°, was colorless; second fraction 47–48°, slightly yellow; third fraction 48–58°, distinctly yellow. A small amount of brown liquid which remained in the flask at the end of distillation was discarded.

Preparation C was the product of distillation at 18 mm. of Hg, and was slightly yellow.

Preparation D stood in the ice box 35 days at 10° after being distilled with diminished pressure.

Preparation E was distilled at 1 mm. of Hg into a receiver sur-

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[1] Copper-lime filtrates should be acidified with sulfuric acid before addition of the H$_2$SO$_4$-Na$_2$SO$_4$-MnSO$_4$ mixture.

[2] The author wishes to express his appreciation to Dr. E. S. West for instruction and assistance in the micro analyses.

rounded with freezing mixture and was kept cold until weighed and diluted.

Lithium Pyruvate Preparations

Preparations A and B were prepared by adding solid Li_2CO_3 in small portions to hot 15 per cent aqueous solutions of pyruvic acid (redistilled some weeks previously) until all the acid was neutralized. After being reacidified with 2 to 3 drops of acid the solution was concentrated by boiling (it assumed a slight yellow color and caromel odor) and placed in the cold room to crystallize. The crystals were freed of mother liquor on a glass filter, washed several times with cold water (until washings were no longer acid to litmus), and dried *in vacuo* over H_2SO_4.

Preparation C was prepared from Preparation B by recrystallization from the least volume of water, weakly acidified with pyruvic acid, which would dissolve it with boiling.

Preparation D was prepared similarly as Preparations A and B and recrystallized twice.

Preparation E was a second crop of crystals obtained by concentration of the mother liquor from Preparation D.

Preparation F was prepared similarly as Preparations A and B, using pyruvic acid Preparation E immediately after its distillation.

Preparation G was prepared from pyruvic acid which had stood 5 days in the ice box after redistillation. Concentration was accomplished with the water pump at 45–50°. At no time during its preparation was the material subjected to higher temperature.

Preparation H was prepared by low temperature distillation of pyruvic acid into an ice-cold suspension of Li_2CO_3. After an excess of acid distilled over, the material was digested at 0° with frequent shaking for 2 days. It was then evacuated repeatedly to remove CO_2 and returned to the cold room for digestion overnight. The crystals were washed and dried as usual.

DISCUSSION

Methods—The reduction procedure is flexible as regards quantity of pyruvic acid and volume of solution reduced (equal recovery with 75 to 0.5 mg.). It is rapid, being complete with 40 cc. of solution in 1.5 minutes (the shortest period investigated). The

rate of reduction probably varies with the volume of the sample, although a 150 cc. sample is completely reduced by the usual procedure. The formation of lactide or methylglyoxal, suggested by Krishna and Sreenivasaya (39) as possibly being responsible for the low results obtained by them, is excluded by the fact that heating with alkali subsequent to reduction leads to no increased yield of lactic acid.

The modified Friedemann-Cotonio-Shaffer lactic acid method gives consistent recoveries of 97 to 98 per cent with Zn or Li lactate.

TABLE I

Comparison of Samples of Pyruvic Acid

Experiment No.	Preparation	Alkali titration*		Iodoform reaction		Reduction to lactate	
		0.1 N NaOH	Per cent of theory	0.1 N I₂	Per cent of theory	0.005 N I₂	Per cent of theory
		cc.		*cc.*		*cc.*	
108	A (not redistilled)	13.70	97.1	10.55	87.8	8 56	85.6
		26.18	97.2	13.13	87.6	8.52	85.2
		26.12	97.6			8.66	86.6
125	B (redistilled)						
	1st fraction	20.70	97.3	11.82	92.4	19.85	93.2
	2nd "	22.68	99.7	12.86	94.5	21.55	94.8
	3rd "	20.60	100.1	11.66	94.5	19.18	93 2
200	C (redistilled)	27.65	100.2	31.70	95.3		
V, 1-a	D "	27.13	98.2	61.80	93.3		
V, 1-b	E "	27.40	99.5	62.30	95.2		

* Phenolphthalein.

The iodoform reaction as carried out by us is rapid, the iodine consumption being as great in 5 as in 20 minutes. This rapidity is accomplished by carrying out the reaction in approximately 2 N NaOH. The slowness of the reaction as described by Wieland (30), Cook (35), and Hatcher and Mueller (32) is due to their use of more dilute alkali. Great excess of iodine over the stoichiometric requirement is unnecessary according to our experience.

Pyruvic Acid—The effect of redistilling pyruvic acid on alkali titer, iodine consumption (in alkaline solution), and lactate yield by reduction is illustrated by the data in Table I. Since the three

fractions of Preparation B represent all but an insignificantly small amount of Preparation A, the increased recoveries notable in *each* fraction must result from depolymerization during distillation. Using the iodoform reaction as a criterion of purity of various preparations, we have not obtained pyruvic acid which showed greater than 95 per cent theoretical iodine uptake, even after low temperature distillation and collection at $-10°$. Hatcher and Hill (29) obtained a maximum recovery of 95 per cent by the iodoform reaction, although Wieland (30) found the concentration by weight, iodine consumption, and alkali titration of "pure" pyruvic acid to agree perfectly. Cook (35) confirmed Wieland's findings. In neither case, however, was the method of preparing "pure" pyruvic acid described. Undoubtedly the 20 per cent deficiency in recovery of pyruvic acid, observed by Krishna and Sreenivasaya, is due to the use of old polymerized material, and the improvement of recovery (from 85 to 94 per cent) occasioned by heating 2 hours on a boiling water bath with the reductant (Zn-Cu-$NaHSO_4$), reported by Kendall and Friedemann, may probably be similarly explained. According to our experience, heating during reduction is unnecessary if freshly redistilled acid is employed. Furthermore, we have kept dilute solutions (0.05 M) of pyruvic acid in and out of the ice box as long as 4 months without deterioration.

Lithium Pyruvate—In early attempts to prepare a lithium salt of pyruvic acid, concentration was accomplished by boiling, and, although the several samples agreed well as regards lithium content, iodine consumption, and lactate yield, theoretical values were not obtained (see Table II). By preparation and concentration at lower temperature, however, Preparation G was obtained which gave 98.5 per cent of the theoretically expected lactic acid yield and 97.5 per cent of the calculated iodine consumption. The elementary analyses (H excepted) agree within 1 per cent of theory, 1 molecule of water being assumed, although no loss in weight occurs during 24 hours heating at 80°. Preparation H, which represents an attempt to prepare the salt at low temperature in the presence of a minimum of polymerized acid, is apparently of the same degree of purity as Preparation G. It is our opinion that the 1.5 per cent "loss" is due to the presence of a small amount of a Li salt of a polymer of pyruvic acid, which is not re-

duced to lactate and which does not react with iodine in alkaline solution, rather than to some defect in the reduction procedure.

Recovery from Blood—Estimation of pyruvic acid in biological material is accomplished by determination of lactic acid on deproteinized filtrates before and after reduction. Mercuric chloride (Schenck) and trichloroacetic acid may be used as precipitating agents with 100 per cent recovery. The latter agent has the disadvantage of introducing a considerable blank and of interfering with reduction. Its use will require further study. The Schenck procedure is recommended. Use of zinc sulfate (Somogyi (42))

TABLE II

Comparison of Samples of Lithium Pyruvate

Experiment No.	Preparation	Iodoform reaction		Reduction to lactate		Elementary analysis		
		0.1 N I₂	Per cent of theory	0.005 N I₂	Per cent of theory	Li	H	C
		cc.		*cc.*		*per cent*	*per cent*	*per cent*
162	A			42.38	91.3			
184	B	12.00	95.4	19.16	91.3			
188	C	11.72	93.2	19.56	93.4	6.03		
207, a	D	25.25	94.2	32.90	92.1	5.98		
IV, 3	E	25.20	94.1	16.55	92.6			
V, 2	F	25.12	93.8					
V, 3	G	26.12	97.5	17.23	96.5*	6.15	4.74	31.8
V, 6	H	25.93	96.8					
	Theory					6.20	4.50	32.1

* 98.5 per cent recovery, when corrected for incomplete recovery by the lactic acid method.

and tungstic acid (Folin-Wu) as blood precipitants leads to irregular losses. Treatment with copper-lime applied *after* reduction and filtration leads to no loss, and is desirable with blood or tissue extracts.

We have compared the rapid reduction procedure with the slower procedure of Kendall and Friedemann on blood filtrates containing pyruvic acid (1), and no difference was detected. It follows that pyruvic acid does not undergo polymerization in the presence of blood (dog). Its fate in other tissues is being investigated.

Methylglyoxal and HCN are interfering substances of some importance.[3] We have not been able to dissipate the interference of the former. The latter does not interfere if reduction is accomplished at 100°, with the procedure of Kendall and Friedemann. Glucose in high concentration is without effect during reduction; it should be removed by copper-lime precipitation after the reduction.

SUMMARY

The effect of redistillation of pyruvic acid on alkali titer, iodine consumption in alkaline solution, and reduction to lactate is reported. The preparation and composition of a lithium salt of pyruvic acid which serves as a suitable substance for quantitative test of methods for pyruvic acid determination are described. The conditions are described for the rapid and apparently complete reduction (in the cold) of pyruvic acid to lactic acid by the Zn-Cu couple in sulfuric acid solution and subsequent determination of the latter by the Friedemann-Cotonio-Shaffer method.

This work has been carried out under the direction of Professor P. A. Shaffer.

BIBLIOGRAPHY

1. Wendel, W. B., *Proc. Soc. Exp. Biol. and Med.*, **27**, 624 (1929–30). Wendel, W. B., and Shaffer, P. A., *J. Biol. Chem.*, **87**, p. xx (1930).
2. Kendall, A. I., and Friedemann, T. E., *J. Infect. Dis.*, **47**, 176 (1930).
3. Berzelius, J. J., *Ann. Physik u. Chem.*, **36**, 1 (1835).
4. Simon, L., *Compt. rend. Acad.*, **125**, 534 (1897).
5. Quastel, J. H., *Biochem. J.*, **18**, 365 (1924).
6. Posternak, *Compt. rend. Soc. physiq. hist. nat. Genève*, **44**, 519 (1927), cited by Cook (35).
7. Alvarez, E. P., *Chem. News*, **91**, 209 (1905).
8. Garzarolli-Thurnlackh, K., *Monatsh. Chem.*, **20**, 467 (478) (1899).
9. Anderson, J. A., Peterson, W. H., and Fred, E. B., *Soil Sc.*, **25**, 123 (1928).

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[3] HCN also interferes with the lactic acid determination, behaving towards bisulfite similarly as acetaldehyde. This interference is overcome by acidifying the sample containing lactic acid and HCN with H_2SO_4, adding water to 60 or 100 cc., and boiling vigorously (in the lactic acid oxidizing flask) for a few minutes before connecting with the distillation-aeration apparatus.

10. Fischer, E., *Ber. chem. Ges.*, **17**, 572 (1884).
11. Fischer, E., and Jourdan, F., *Ber. chem. Ges.*, **16**, 2241 (1883).
12. de Jong, A. W. K., *Rec. trav. chim. Pays-Bas*, **19**, 280 (1900), cited by Kendall and Friedemann (2).
13. MacLean, I. S., *Biochem. J.*, **7**, 611 (1913).
14. Simon, L. J., and Piaux, L., *Bull. Soc. chim. biol.*, **6**, 477 (1924).
15. Hahn, A., Fischbach, E., and Haarmann, W., *Z. Biol.*, **88**, 89 (1929).
16. Hyde, E., *Ber. chem. Ges.*, **32**, 1810 (1899).
17. Neuberg, C., and Kobel, M., *Biochem. Z.*, **216**, 493 (1929).
18. Simon, E., and Neuberg, C., *Biochem. Z.*, **232**, 479 (1931).
19. Kostytschew, S., and Soldatenkov, S., *Z. physiol. Chem.*, **168**, 124 (1927); **176**, 287 (1928).
20. Beilstein, F., and Wiegand, E., *Ber. chem. Ges.*, **17**, 840 (1884).
21. Fernbach, A., and Schoen, M., *Compt. rend. Acad.*, **158**, 1719 (1914).
22. Holleman, A. F., *Chem. Zentr.*, **2**, 194 (1904).
23. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **17**, 443 (1914).
24. Bleyer, B., and Braun, W., *Biochem. Z.*, **183**, 310 (1927).
25. Mazé, P., and Ruot, M., *Compt. rend. Soc. biol.*, **79**, 706 (1916).
26. Denis, W., *Am. Chem. J.*, **38**, 561 (577) (1907).
27. Evans, W. L., and Witzemann, E. J., *J. Am. Chem. Soc.*, **34**, 1086 (1096) (1912).
28. Lieben, F., *Biochem. Z.*, **135**, 240 (1923).
29. Hatcher, W. H., and Hill, A. C., *Tr. Roy. Soc. Canad.*, **22**, sect. 3, 211 (1928).
30. Wieland, H., *Ann. Chem.*, **436**, 229 (1924).
31. Neuberg, C., *Biochem. Z.*, **43**, 500 (1912).
32. Hatcher, W. H., and Mueller, W. H., *Tr. Roy. Soc. Canad.*, **23**, sect. 3, 35 (1929).
33. Warburg, O., Kubowitz, F., and Christian, W., *Biochem. Z.*, **227**, 245 (1930).
34. Mendel, B., Bauch, M., and Strelitz, F., *Klin. Woch.*, **10**, 118 (1931).
35. Cook, R. P., *Biochem. J.*, **24**, 1526 (1930).
36. Wislicenus, J., *Ann. Chem.*, **126**, 225 (1863).
37. Debus, H., *Ann. Chem.*, **127**, 332 (1863).
38. Laufberger, V., *Biochem. Z.*, **181**, 220 (1927).
39. Krishna, B. H. R., and Sreenivasaya, M., *Biochem. J.*, **22**, 1169 (1928).
40. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, **73**, 335 (1927).
41. West, E. S., *J. Biol. Chem.*, **92**, 483 (1931).
42. Somogyi, M., *Proc. Soc. Exp. Biol. and Med.*, **26**, 353 (1928–29).

A COLORIMETRIC METHOD FOR THE DETERMINATION
OF ALLANTOIN*

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INTRODUCTION

Since the publication, in 1908, of Wiechowski's (1) method for
the determination of allantoin, various attempts have been made
to simplify the method and to increase its accuracy. These
attempts have not met with much success.

The Wiechowski method depends upon the precipitation of
allantoin by a mercuric acetate reagent after the urine has been
freed from interfering substances by a very complicated and time-
consuming procedure. The mercury-allantoin precipitate ob-
tained is decomposed by hydrogen sulfide, and the allantoin re-
crystallized or a Kjeldahl nitrogen determination made on the
precipitate. Handovsky (2), in 1914, introduced the use of a
known amount of standard mercuric acetate-sodium acetate re-
agent. In his modification, the mercury-allantoin compound is
filtered off and the mercury remaining in the filtrate is titrated
against standard ammonium thiocyanate. This involves a very
difficult end-point. Unfortunately, small errors in this titration
make considerable differences in allantoin values.

In more recent literature (3–6), methods have appeared which
are based upon the hydrolysis (by acid, alkali, or enzyme) of
allantoin. Certain products of hydrolysis, such as urea, hydan-
toin, glyoxylic acid, and oxalic acid, are determined, and from
these the amount of allantoin is estimated. These methods are
based upon the hypothesis that the hydrolysis of allantoin pro-
ceeds in a clear cut, orderly manner, and that the so called end-

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products of hydrolysis represent quantitative values. There is wide-spread disagreement as to the exact course and mechanism of these reactions, as well as to the products involved. Until proof is established concerning the true character of these allantoin hydrolyses, such methods are open to considerable doubt.

All of the published methods for allantoin determination require from 6 to 24 hours to perform. Many of them are probably inaccurate, at best giving not more than approximate values for allantoin. Because of the importance of animal purine metabolism, the need for a more accurate and rapid method has become urgent. The present investigation was undertaken in the endeavor to provide such a method.

Folin and Svedberg (7), in working with various copper reagents for carbohydrate determination, developed an ammoniacal copper reagent which is practically unaffected by urinary sugar, but which is reduced by nitrogenous compounds such as creatine, creatinine, and allantoin. It is the use of this reagent which makes the following colorimetric method possible.

Colorimetric Determination of Allantoin

General Outline

5 cc. of animal urine are treated with an excess of 30 per cent phosphotungstic acid, followed by an excess of saturated basic lead acetate solution, and 5 per cent sulfuric acid. This treatment removes interfering substances. The procedure is carried out in the same 50 cc. centrifuge tube. After the addition of each reagent, the tube is gently rotated to insure proper mixing, and the mixture is centrifuged until perfectly clear. 2 cc. of this liquid are pipetted into a Folin-Wu sugar tube together with 2 cc. of Folin ammoniacal copper regent which is reduced by allantoin. This is then heated in a boiling water bath for 10 minutes, cooled, and 2 cc. of acid molybdate reagent added. The color obtained is read against a 1 mg. allantoin standard. Recoveries of allantoin added to rat urine range from 90 to 100 per cent. Values obtained colorimetrically check with those obtained by use of the mercury-allantoin reagent on the same solution. 2 hours are required for the complete determination, as against the 10 or 12 hours required by the Wiechowski-Handovsky method now in general use.

Reagents Required

Phosphotungstic Acid—The phosphotungstic acid solution is made up in the centrifuge tube directly before using, as the solution seems to deteriorate rapidly on standing. Eight different lots of C.P. phosphotungstic acid obtained from four leading manufacturers varied greatly in their precipitating power, and gave inconsistent results. It is therefore necessary to prepare pure phospho-24-tungstic acid.

The preparation of the pure acid is essentially according to Wu (8) and is as follows: Dissolve 100 gm. of $Na_2WO_4 \cdot 2H_2O$ in about 100 cc. of water with the aid of heat. Add 10 cc. of 85 per cent H_3PO_4 and then 80 cc. of concentrated HCl. Cool. After 4 hours or more, filter on a Buchner funnel and suck as dry as possible. Redissolve the precipitate in 120 cc. of H_2O, pour the solution into a liter separatory funnel, add about 90 cc. of ether, and then add 40 cc. of concentrated HCl. Shake. After standing a few minutes, there should be three layers of liquid. The lowest layer contains nearly all the complex acid. If there are only two layers, more ether must be added and the mixture shaken again. Transfer the lowest layer to another separatory funnel, add about 120 cc. of water, and shake vigorously; then add 50 cc. of ether and finally 50 cc. of concentrated HCl. After standing, the lowest layer, which should be perfectly clear, is transferred to a crystallizing dish. Add 30 cc. of H_2O and 1 drop of liquid bromine and evaporate on a steam bath. The solution should be greenish in color. If the slightest trace of dust or organic matter is present, a pinkish color develops; and 1 or 2 drops more of liquid bromine must be added to oxidize this foreign material.

Evaporate on the steam bath until crystals begin to form on the surface. Let stand overnight. The crystals obtained are sucked as dry as possible on a large Buchner funnel. After air-drying for 1 week, powder the crystals and keep in an amber glass container. This phosphotungstic acid should dissolve instantly to give a perfectly clear, practically colorless solution.

Basic Lead Acetate Solution (9)—Dissolve 180 gm. of lead acetate in about 700 cc. of distilled water, with boiling. Add 110 gm. of lead oxide (litharge) and boil for $\frac{1}{2}$ hour with occasional stirring. Cool, filter, and add sufficient distilled water to the filtrate to make the weight 1 kilo.

Folin Ammoniacal Copper Solution (7)—Dissolve 100 gm. of ammonium sulfate in about 400 cc. of water and filter into a volumetric liter flask. 100 cc. of 10 per cent sodium hydroxide are then added, 12 gm. of sodium tartrate, and finally a solution of 5 gm. of copper sulfate. Dilute to volume and mix. This reagent will not give a blank for months, if kept in the dark in well filled, tightly stoppered, amber glass bottles. The bottles should be of small volume.

5 Per Cent Sulfuric Acid (by Weight).

Folin Acid Molybdate Reagent (10)—Prepare a stock solution of 30 per cent brominated sodium molybdate as follows: Dissolve 300 gm. of sodium molybdate in water and make up to 1 liter. The solution is slightly turbid. Add 2 or 3 drops of liquid bromine and let stand overnight. Transfer 500 cc. of the clear supernatant liquid to a liter flask and add, with stirring, 225 cc. of 85 per cent phosphoric acid. Then add 150 cc. of cool sulfuric acid (25 volumes per cent). The bromine which is liberated is removed by aeration. Add 75 cc. of 99 per cent acetic acid, mix, and dilute to 1 liter.

Allantoin Standard—Dissolve 100 mg. of allantoin in about 50 cc. of water with the aid of heat, but do not allow to boil. Cool, transfer to a 100 cc. volumetric flask, and dilute to volume. Cover with toluene. Such a standard will keep for about 2 weeks at room temperature without deterioration. Without toluene, a loss of 1 per cent of the allantoin is noted after standing 1 week. Givens (11) reports a loss of 1.7 per cent after 90 days.

Procedure

Transfer 1.5 gm. of phosphotungstic acid to a 50 cc. centrifuge tube and add 5 cc. of water. Rotate gently to insure solution; then add 5 cc. of animal urine. Centrifuge immediately, place the tube in a refrigerator for $\frac{1}{2}$ hour, then centrifuge again until perfectly clear. The addition of a crystal of phosphotungstic acid should not cause further precipitation. Add 5 cc. of basic lead acetate solution which precipitates the excess phosphotungstic acid as well as the remaining interfering substances. Centrifuge the mixture, then treat with 5 cc. of 5 per cent sulfuric acid to remove the excess lead, and then centrifuge until perfectly clear. Pipette 2 cc. of the resulting water-clear liquid into a Folin-Wu

sugar tube, neutralize with 5 per cent sodium hydroxide, and then add 2 cc. of Folin ammoniacal copper reagent. Place the tubes in a rapidly boiling water bath for 10 minutes, cool, then add 2 cc. of acid molybdate reagent. Dilute the tubes to volume and read against a 1 mg. allantoin standard.

Remarks

The above method was designed primarily for use with rat urines, but has been used successfully with other urines. The method depends upon a proper balancing of reagents involved. The amount of phosphotungstic acid used above is in slight excess for normal urines. The lead is sufficient to precipitate the excess phosphotungstic acid and certain reducing materials, and the 5 cc. of sulfuric acid completely remove the excess lead. However, when certain urines, e.g. from a high protein diet, are used, the amount of phosphotungstic acid is not in excess. When such a urine is encountered, simply add more solid phosphotungstic acid until no further precipitation occurs (usually, 0.2 gm. will suffice). To insure complete removal of lead, in such a case, after treatment with the 5 cc. of sulfuric acid an additional cc. should be added to make sure that there is no further formation of lead sulfate. It is only in rare instances that urines are encountered which require the above variations in procedure. Of course, initial dilution of such a urine or the use of a smaller quantity entirely circumvents the difficulty. When a urine contains large amounts of allantoin, as little as 1 cc. may be used. On the other hand, where a urine is low in allantoin, 10 cc. should be employed instead of 5. In such a case, the solid phosphotungstic acid may be added directly to the urine, instead of first dissolving it in water.

It has been claimed that certain amino acid-phosphotungstate precipitates dissolve when allowed to remain in excess phosphotungstic acid for any length of time. In order to minimize this possibility, the precipitated urine is centrifuged before it is placed in the refrigerator. The use of the refrigerator is not a necessary part of the method. It merely cuts the time of complete phosphotungstate precipitation to a minimum.

Sometimes particles of lead sulfate remain floating on the surface of the liquid or adhere to the walls of the tube and it is diffi-

cult to pipette the liquid without contamination.   To avoid this difficulty, the liquid is transferred to another tube by decantation, and centrifuged for a minute or two.

Comparison of the Wiechowski-Handovsky and Colorimetric Methods

The Handovsky modification of the Wiechowski procedure for allantoin determination is the most generally accepted method. For this reason, it was chosen as a basis for comparison with the colorimetric method.   During the past 2 years, the writer has had occasion to run a great number of these Wiechowski determinations.   The enormous, heavy precipitates encountered have been centrifuged off, instead of being filtered.   It was felt that this would increase the accuracy of the method, and this was the only deviation from the regular Wiechowski procedure.   In spite of every precaution, it was impossible to obtain consistent results by the Wiechowski-Handovsky method, even for duplicate determinations.   There was wide discrepancy between the results obtained by this method and those by the colorimetric method. In general, the higher results were obtained by the latter.

In Table I are shown results obtained by the Wiechowski-Handovsky and colorimetric methods.   The colorimetric values vary from those of Wiechowski from −24.0 per cent to +31.7 per cent.

Recovery of Added Allantoin

Allantoin added to rat urines may be recovered satisfactorily by the colorimetric method.   Consistent recoveries of from 90 to 100 per cent are obtained, the average for twenty-five recoveries being 97.2 per cent.   If the recovery of added allantoin is taken as a criterion of the accuracy of the method, the colorimetric method is vastly superior to the Wiechowski-Handovsky procedure.   Recoveries by the latter method range from 20 to 87 per cent, the majority being in the neighborhood of 70 per cent.   It is reasonable to assume that the method which gives the higher percentage recovery is the more nearly accurate one.

In Table II are given the recoveries of added allantoin by the colorimetric method and the low recoveries obtained by the Wiechowski-Handovsky method from the same urines.

In Table III are given allantoin recoveries for urines containing

TABLE I

Comparison of Allantoin Values Obtained by Two Methods on the Same Urine

Rat urine No.	Allantoin per cc.		Percentage difference
	Wiechowski-Handovsky method	Colorimetric method	
	mg.	mg.	
1	3.36	3.18	−5.3
2	3.45	3.38	−2.0
3	2.40	2.73	+13.7
4	2.93	2.90	+1.0
5	2.70	2.51	−7.5
6	3.10	2.62	−15.5
7	2.58	2.66	+3.1
8	1.77	2.05	+15.8
9	2.41	1.83	−24.0
10	2.04	2.52	+23.5
11	1.69	2.12	+25.4
12	1.67	2.20	+31.7

TABLE II

Recovery of Allantoin Added to Urine

Method	Specimen		Allantoin per 100 cc. urine	Allantoin added to 100 cc. urine	Total allantoin found per 100 cc. urine	Allantoin recovered
			mg.	mg.	mg.	per cent
Colorimetric	Rat Urine	9	183.8	100	283.9	100.0
	" "	10	206.0	100	301.2	95.2
	" "	11	252.4	100	349.4	97.0
	" "	12	212.0	100	308.8	96.8
	" "	13	220.0	100	310.0	90.0
	Dog urine		147.2	100	240.8	93.6
	Monkey urine		52.4	100	144.8	92.4
Wiechowski-Handovsky	Rat Urine	9	241.8	60	289.4	79.3
	" "	10*	83.6	60	101.1	29.1
	" "	11	204.7	165	313.3	65.8
	" "	12	168.5	100	255.2	86.7
	" "	13	166.6	165	292.8	76.4
	Dog urine		143.8	100	231.1	87.3
	Monkey urine		78.4	100	162.8	84.4

* This urine required a great excess of silver nitrate which was removed with difficulty.

varying amounts of allantoin, as well as other reducing substances. Recoveries range from 90 to 102 per cent, and tend to show that allantoin recoveries by the colorimetric method are independent of the kind of animal urine used, as well as of the concentration of reducing substances. The recovery is as good for rabbit urine containing only a small amount of allantoin as it is for rat urines which contain large amounts of allantoin.

TABLE III

Recovery of Allantoin Added to Urine Containing Varying Amounts of Allantoin.   Colorimetric Method

Urine	Allantoin per cc.	Total allantoin excreted per 24 hrs.	Recovery of added allantoin
	mg.	mg.	per cent
Dog, normal......................	1.47	260.4	93.6
" diabetic (1.7 per cent sugar)	2.06	206.0	98.4
" fasting (1.45 " " crea-			
tine)...........................	4.66	326.2	100.0
Dog, fasting......................	2.19	481.7	98.3
Rabbit...........................	0.39	128.7	102.0
"	0.53	136.2	94.1
Monkey..........................	0.81	181.8	90.8
"	0.78	184.2	90.2
Rat*.............................	7.65	66.3	93.6
" †.............................	6.92	73.8	102.0
" ‡.............................	8.57	97.1	96.2

* The diet contained 72.5 per cent dried liver residue and 2.5 per cent nucleic acid.

† The diet contained 70.0 per cent dried liver residue and 5.0 per cent nucleic acid.

‡ The diet contained 65.0 per cent dried liver residue and 10.0 per cent nucleic acid.

Creatine and sugar, while not completely removed by the colorimetric procedure, are present in such small amounts that their reduction may be considered negligible. A 0.1 per cent creatine solution gives a faint blue coloration with the copper reagent, whereas a 0.1 per cent creatinine solution gives a stronger color than a 0.1 per cent allantoin solution.

It is realized that higher allantoin recoveries do not constitute final proof of the accuracy of the colorimetric method. Inasmuch

as this method gives, in general, higher allantoin values than the Wiechowski method, it was felt that proof must be given that reduction of the copper reagent comes from allantoin only, and not from any other substances which might be present.

In order to furnish this proof, the following experiments were devised, in which the allantoin was determined on the same filtrate, not only by copper reduction, but also by mercury precipitation. Allantoin may be quantitatively recovered from its solutions by precipitating the mercuric acetate-sodium acetate reagent. After filtering off the mercury-allantoin precipitate, the excess mercury remaining in the filtrate is titrated with standard ammonium thiocyanate and the allantoin calculated from this titration. In order to apply this Handovsky determination to the final liquid obtained by the colorimetric procedure, it is first necessary to remove the chlorides. Chlorides interfere with the ammonium thiocyanate titration, and must be removed by treatment with silver nitrate, and the excess silver precipitated by hydrogen sulfide.

The procedure devised for this purpose is identical with the regular colorimetric procedure, save that three times the quantity of urine and various reagents are used, so that the final volume is 60 cc. instead of 20. A 100 cc. centrifuge tube is used instead of the 50 cc. size. The chlorides are removed from the 60 cc. volume by treatment with 15 cc. of 0.1 per cent silver nitrate solution. The precipitate is centrifuged off; the liquid is transferred to a beaker, and the excess silver removed by hydrogen sulfide. After filtering off the precipitated sulfides and aerating to remove any hydrogen sulfide, the filtrate is ready for the dual determination.

2 or 3 cc. of this filtrate are used for the colorimetric determination. For the Handovsky determination, 50 cc. of the filtrate are pipetted into a 100 cc. volumetric flask, carefully neutralized, and 40 cc. of the standard mercuric acetate-sodium acetate reagent added, and the contents of the flask diluted to volume. From this point, the regular Handovsky procedure is followed.

In Table IV are shown the results obtained by the two methods. The agreement is quite close and within the limits of error of the ammonium thiocyanate titration. Thus, by two entirely different means, the same allantoin values are obtained, showing that

in the colorimetric procedure the urine is freed from all reducing material other than allantoin.

When allantoin was added to the urine, recoveries were practically identical for the two determinations on the same filtrate. It is interesting to note here, that the percentage recoveries obtained are 10 to 15 per cent lower than by the regular colorimetric procedure. Inasmuch as the silver nitrate-hydrogen sul-

TABLE IV

Comparison of Allantoin Values Obtained by the Two Methods on Same Filtrate. Filtrate from 15 Cc. of Rat Urine. Colorimetric Procedure

Urine	Added allantoin	Colorimetric determination	Wiechowski-Handovsky Hg precipitation, NH₄SCN titration
	mg.	*mg.*	*mg.*
Rat 100................		38.6	37.4
" 101...........................		33.2	31.8
" 102....................	15	49.7	50.5
" 103...........................		38.0	37.8
" 104......	15	55.0	50.7
" 105.......		26.6	26.3
" 106...........................	15	40.7	37.9
" 107...........................		25.5	25.3
" 108........................ ...		30.6	31.3
" 109...........................	15	44.4	44.5
" 110...........................		23.9	24.8
" 111...........................		32.5*	33.7*
Dog 1...........................		72.4	70.9
" 2...........................		30.5	31.3
" 3...........................		20.3	21.5
" 4...........................		33.6	34.6

* The filtrate was obtained by the Wiechowski procedure.

fide treatment is the only deviation from this procedure, it suggests one of the sources of loss of allantoin in the Wiechowski-Handovsky determination. It is not the purpose of this paper to point out the various sources of error in the Wiechowski-Handovsky method, but from the writer's experience with this method, the loss of allantoin due to adsorption on the tremendous bulky precipitates of lead-silver sulfides is considerable (see Table II, Urine 10).

Allantoin in Human Urine

By using a modification of his method for animal urine, Wiechowski (12) found minute amounts of allantoin present in human urine, 5 to 12 mg. per liter. (At such a dilution it is almost impossible to precipitate allantoin from pure solution by the mercuric acetate reagent.)

If human urine contains allantoin in such small amounts, it would appear that a better yield would result by employing a modification of the colorimetric procedure for ridding urine of interfering substances. By this modified procedure 25 mg. of allantoin were obtained from a 24 hour specimen of 1205 cc. Specimens of mixed human urines yield 25 and 30 mg. of allantoin per liter. These figures agree closely with those recently published by Fosse (13), using the spectrophotometric method.

The mercury-allantoin precipitates obtained were decomposed with hydrogen sulfide, and the small amount of allantoin recrystallized twice. The melting point determined was 236–237°. Wiechowski reports a melting point of 232° for his recrystallized product. Watt (14) gives 235° as the melting point of pure allantoin. The crystals obtained, while few in number, were of characteristic structure. A solution of these crystals readily reduced the Folin ammoniacal copper reagent.

SUMMARY

A rapid and accurate method for the determination of allantoin is described.

BIBLIOGRAPHY

1. Wiechowski, W., *Beitr. chem. Physiol. u. Path.*, **11**, 109 (1908).
2. Handovsky, H., *Z. physiol. Chem.*, **90**, 211 (1914).
3. Christman, A. A., *J. Biol. Chem.*, **70**, 173 (1926).
4. Fosse, R., Brunel, A., and de Graeve, P., *Compt. rend. Acad.*, **188**, 1418, 1632 (1929).
5. Allen, F. W., and Cerecedo, L. R., *J. Biol. Chem.*, **93**, 293 (1931).
6. Fosse, R., Brunel, A., and Thomas, P. E., *Compt. rend. Acad.*, **192**, 1615 (1931).
7. Folin, O., and Svedberg, A., *J. Biol. Chem.*, **70**, 418 (1926).
8. Wu, H., *J. Biol. Chem.*, **43**, 197 (1920).
9. Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 10th edition, 1888 (1931).
10. Folin, O., *J. Biol. Chem.*, **82**, 88 (1929).

11. Givens, M. H., *J. Biol. Chem.*, **18,** 417 (1914).
12. Wiechowski, W., *Biochem. Z.*, **19,** 378 (1909).
13. Fosse, R., Brunel, A., and Thomas, P. E., *Compt. rend. Acad.*, **192,** 1619 (1931).
14. Watt, H. E., *Pharm. J.*, **99,** 283 (1917).

THE METABOLISM OF SULFUR

XVIII. THE DISTRIBUTION OF URINARY SULFUR IN THE RABBIT AFTER THE ADMINISTRATION OF MONOBROMOBENZENE

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The evidence concerning the ability of the animal organism to effect the synthesis of the phenylmercapturic acids after the administration of the monohalogen substitution products of benzene has been secured in two ways. The most conclusive of the available evidence has been the actual isolation of the mercapturic acids from the urine, but unfortunately this procedure is far from quantitative and the isolation of the acid, although offering unmistakable proof of the synthesis, gives little information as to its extent. The second type of information is to be obtained from a consideration of the changes in the sulfur partition of the urine after the administration of the halogen benzene derivative. This procedure, which has been used extensively by Hele (1) in his studies on mercapturic acid synthesis in the dog, gives quantitative data as to the extent of the synthesis, provided the assumption made is correct; *i.e.*, that the changes in the organic sulfur accurately indicate the extent of the mercapturic acid synthesis. An exact quantitative method for the estimation of the mercapturic acids is urgently needed.

The manner in which the halogen benzene derivatives are metabolized appears to vary with the species of experimental animal. Proof of the synthesis in the dog obtained by both the methods of study discussed is available (1). The pig differs from the dog in that synthesis of the mercapturic acids is not readily effected (2). In the hen, iodobenzene is converted into and excreted as iodophenylglycuronic acid (3). In the rabbit this problem has not been extensively investigated. Rhode (4), concerned with the origin

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of the ethereal sulfates in the rabbit, noted that the increase in ethereal sulfates observed after bromobenzene feeding was less than when cystine was fed with the bromobenzene and suggested that this was evidence of the synthesis of a phenylmercapturic acid.   No analyses in which the organic sulfur of the urine was determined are reported.   Abderhalden and Wertheimer (5), who were able to isolate small amounts of p-bromophenylmercapturic acid from the urine of rabbits after the subcutaneous administration of monobromobenzene, emphasized the nature (primarily the reaction) of the diet as an important factor, since they were able to isolate the acid only from the urine of animals fed an oat diet, a diet which resulted in the production of an acid urine.   In experiments with animals fed a diet of green food, no evidence of the formation of the mercapturic acid could be secured, even when cystine was injected with the bromobenzene.   Nishimura (6) isolated phenylmercapturic acids from the urine of fasting rabbits and of rabbits fed okara after the administration of the monoiodo-, monobromo-, and monochlorobenzene.   More recently Lawrie (7) in Hele's laboratory has concluded as a result of isolation experiments that the rabbit "is able to convert a very small fraction of injected iodobenzene into p-iodophenylmercapturic acid."   In the one experiment reported in which a study of the distribution of urinary sulfur was made, no marked increases in the excretion of organic sulfur were observed after either oral or subcutaneous administration of iodobenzene.   It is apparent from this brief summary that little evidence concerning the extent of mercapturic acid synthesis in the rabbit is available.

Our experiments, which were carried out prior to the appearance of Lawrie's paper, had for their purpose a study of the evidence of mercapturic acid synthesis in the rabbit to be obtained from the determination of the partition of urinary sulfur.   The data secured have been similar to those obtained by Hele (1) and in this laboratory (8) in experiments with the dog, the animal by which the first and most convincing evidence of synthesis was afforded.

<center>EXPERIMENTAL</center>

Male rabbits were maintained on basal diets of 70 gm. of oats and 100 gm. of cabbage and were confined in the usual metabolism cages which permitted quantitative collection of the urine.   Com-

plete 24 hour specimens of urine were secured by removal of the urine from the bladder by gentle pressure on the abdominal wall. The bromobenzene was fed suspended in water through a small stomach tube in all cases. In the fasting experiments because of possible toxicity of the bromobenzene, the preliminary period of fasting was limited to 2 days, in order to maintain the animal in as good physical condition as possible.

Total nitrogen and creatinine were determined by the usual methods. Sulfur determinations were made according to the volumetric methods of Fiske (9), with the use of Jena glass filter crucibles (No. 10 G 3/7) instead of the paper pulp filters recommended by him. In this method, phosphates are removed by a preliminary precipitation with basic magnesium carbonate. Since the magnesium salt of mercapturic acid is not readily soluble in cold water (10), it was necessary to determine whether any organic sulfur from this source was lost in the preliminary precipitation of the Fiske method. A comparison was made of the organic sulfur values in a urine sample after the administration of the bromobenzene by the Fiske method and the usual gravimetric procedure. The values obtained with the volumetric benzidine method were slightly higher than those with the gravimetric barium sulfate method, indicating that no loss of organic sulfur is to be expected if the Fiske procedure is used for the determination of the partition of sulfur in experiments of the type presented here.

The amounts of bromobenzene fed were such that the animals on the basal diet (Table I) showed no untoward reactions with the smaller dose, 1.5 gm. In one experiment (Rabbit 2, Table I), by an error, twice the usual dosage of bromobenzene, 3.0 gm., was fed. In this experiment, the animal was prostrated for nearly 12 hours and consumed very little of the food. The animal appeared normal on the following day and ate as usual. In the experiments with fasting animals, the animals were prostrated for a few hours but recovered rapidly. The urine excreted in the bromobenzene periods in each case appeared smoky, but no protein could be found in any of the samples of urine.

In Table I are presented the results of experiments with a well fed rabbit (Rabbit 1) which received moderate amounts of the benzene derivative. Marked increases in both the ethereal sulfate and organic sulfur fractions were observed on the experimental

TABLE I

Distribution of Urinary Sulfur after Oral Administration of Bromobenzene to a Fed Animal

	Day	Total N	Creat-inine	Total S	Total sulfate S	In-organic sulfate S	Ethe-real sulfate S	Organic S
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Rabbit 1, weight	1	1.68	165	197	158	145	13	39
3.40 kilos	2	1.63	163	188	147	140	7	41
	3	1.70	178	214	183	175	8	31
	4	1.58	168	163	134	131	3	29
	5*	1.90	167	261	136	30	106	125
	6*	1.82	161	233	115	4	111	118
	7	1.79	163	79	38	3	35	41
	8	1.54	173	174	146	135	11	28
	9	1.47	157	190	146	141	5	44
	10	1.41	168	151	114	106	8	37
Days 1–4, 8–10........		1.57	167	182	147	139	8	35
Extra elimination Days 5–6......		+0.58	−6	+130	−43	−244	+201	+173
Rabbit 2, weight	1	1.03	122	143	119	110	9	24
2.40 kilos	2	1 06	118	138	118	109	9	20
	3	1.03	118	132	113	108	5	19
	4†	1.14	122	198	118	11	107	80
	5‡	1.12	128	60	20	1	19	40
	6	1 42	131	77	32	1	31	45
	7	1.09	113	106	86	82	4	20
	8	1.02	116	112	93	89	4	19
Days 1–4, 7–8.........		1 05	117	126	106	100	6	20
Extra elimination Day 4..............		+0.09	+5	+72	+12	−89	+101	+60
" 5....		+0.07	+11	−66	−86	−99	+13	+20

* 1.5 gm. of monobromobenzene were administered orally (= 0.44 gm. per kilo).

† 1.5 gm. of monobromobenzene were administered orally (≈ 0.62 gm. per kilo).

‡ 3.0 gm. of monobromobenzene were administered orally (≈ 1.24 gm. per kilo).

days.   An increase in the total sulfur fraction and a decrease in the total sulfate fraction were also evident.   The increases in the ethereal sulfate sulfur and in the organic sulfur fractions corre-

sponded to the elimination of 0.985 gm. of the benzene derivative fed as ethereal sulfate sulfur and 0.848 gm. as organic sulfur, or on the 2 experimental days, approximately 61 per cent of the bromobenzene administered could be accounted for by the increases in these two sulfur fractions.   If the slight increases in elimination of ethereal sulfates and organic sulfur of the 1st after day (day 7) be added, a recovery of slightly over 66 per cent in the urine appears.   On the day immediately following the last feeding of bromobenzene, the sulfur excretion, particularly the inorganic fraction, was much depressed, although the excretions of total nitrogen and creatinine were similar to those of the control days. This is well demonstrated by the changes in the urinary N : S ratio. This ratio averaged 8.6 for the control days, 7.5 for the experimental days, and 22.6 for the 1st after day.   Marked retentions of sulfur have also been observed in similar experiments with dogs and will be discussed in a subsequent communication in which these experiments will be detailed.

The data for Rabbit 2 are similar to those just discussed.   However, in this experiment, the dosage of bromobenzene was doubled through an error on the 2nd experimental day and, as already mentioned, the animal showed symptoms of marked toxicity. Although on the 1st experimental day, the distribution of sulfur was similar to that of the preceding experiment, despite the larger amount of bromobenzene administered on the 2nd experimental day, there was little evidence of synthesis of either ethereal sulfates or mercapturic acid and the general level of sulfur excretion was greatly depressed, to a level even lower than that of the 1st after day.   This diminished elimination did not involve the excretion of either total nitrogen or creatinine.   On the 1st experimental day, the increased elimination of ethereal sulfate and organic sulfur was equivalent to 0.789 gm. of bromobenzene or about 52 per cent of that ingested.   On the 2nd day, only 0.162 of the benzene derivative or less than 6 per cent of the amount fed could be accounted for by the increases in these sulfur fractions.   With the increase in dosage (and accompanying toxicity?), the changes in the sulfur distribution were less marked and indicated little if any synthesis.   It is, of course, possible that failure of absorption may have occurred as a result of gastrointestinal irritation produced by the larger dosage.   On this day, a marked odor of bromobenzene

was noted in the expired air shortly after feeding (1.5 to 2 hours). A similar observation has been made by Coombs (11) after the oral administration of fluorobenzene to a dog. This is suggestive of the excretion of bromobenzene or a catabolite through the lungs.

The experiments with fasting animals were similar to those just discussed, except that the changes in ethereal sulfate and organic

TABLE II

Distribution of Urinary Sulfur after Oral Administration of Bromobenzene to a Fasting Animal

Day	Total N	Creatinine	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S	Total N	Creatinine	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S
	Rabbit 3, weight 2.20 kilos							Rabbit 4, weight 1.80 kilos						
	gm.	mg.	mg.	mg.	mg.	mg.	mg.	gm.	mg.	mg.	mg.	mg.	mg.	mg.
1	0.83	118	39	34	29	5	5	0.76	81	51	46	33	13	5
2	1.05	110	43	33	31	2	10	1.06	73	77	71	60	11	6
3*	0.99	112	85	42	2	40	43†	1.93	72	152	101	59	42	51
4*	1.09	112	55	8	2	6	47†	2.86	76	201	141	101	40	60
5	1.28	112	18	3	2	1	15	2.56	69	175	164	156	8	11
6‡	0.91	107	2	2	1	1	0	2.29	45	160	149	141	8	11

* Rabbit 3 received 1.5 gm. of monobromobenzene orally ($\approx$ 0.68 gm. per kilo); Rabbit 4 received 0.996 gm. of monobromobenzene orally ($\approx$ 0.55 gm. per kilo).

† With the excretions of the first 2 days as a basis for normal, the extra sulfur excretions (mg.) on days 3 and 4 are: total sulfur, +44 and +14; total sulfate sulfur, +8 and −26; inorganic sulfate sulfur, −28 and −28; ethereal sulfate sulfur, +36 and +2; organic sulfur, +35 and +40.

‡ The final weight of Rabbit 3 was 1.90 kilos. Rabbit 4 lost weight rapidly; final weight, 1.15 kilos.

sulfur excretions were less marked. With Rabbit 3 (Table II) only 18 per cent of the ingested bromobenzene could be accounted for by the increased elimination of these sulfur fractions on the 2 experimental days, a percentage which is in marked contrast to the greater recovery shown in Table I. The increases in the sulfur fractions of the urine of Rabbit 4 are similar to those of the other fasted animal. 38 per cent of the amount of bromobenzene fed could be accounted for by the increase in sulfur fractions concerned

in the metabolism of the benzene.   With Rabbit 3 on the 2nd ex-
perimental day, there was a marked depression of total sulfur and
total sulfate excretion similar to that recorded for Rabbit 2,
following the administration of a large amount of bromobenzene.
The marked retention of sulfur, especially sulfate sulfur, on the
1st after day is seen here also.   Rabbit 4 (Table II) lost weight
much more rapidly than the others and any effect of the bromo-
benzene feeding on the elimination in the after period is obscured
by the greater excretion of products of metabolism associated
with the tissue breakdown.   The N:S ratio despite the higher
nitrogen level was the same in the after period as in the fore period.

<div align="center">DISCUSSION</div>

The experiments have demonstrated uniformly an increase in
the organic and ethereal sulfate sulfur fractions of the urine and a
decrease in the inorganic sulfate sulfur fraction after oral adminis-
tration of bromobenzene.   Since the possibility of a limited syn-
thesis of the phenylmercapturic acids in the organism of the rabbit
has been demonstrated by others (5–7) and since the sulfur parti-
tions in the urine are comparable to those in similar experiments
with the dog (1, 8), we believe that the chemical processes by which
the halogen benzene derivatives are metabolized in the rabbit are
similar to those which occur in the organism of the dog, in which
this synthesis was first demonstrated.

In the experiments of Lawrie (7) similar to ours, no increases in
the excretion of organic sulfur were observed after feeding 5.4 gm.
of iodobenzene to a rabbit of 2.5 kilos of body weight, *a dosage of
more than 2.0 gm. per kilo of body weight.*   We believe, in the light
of our own experience, that his conditions of experimentation were
not favorable for the synthesis.   We have observed that with the
greater dosages of bromobenzene, there were less marked changes in
the sulfur partition and that the total excretion of sulfur was de-
pressed.   This condition appears in Lawrie's chart, particularly on
the day of the subcutaneous administration of the second dose of
iodobenzene, when there is to be noted a marked depression of both
inorganic sulfate and organic sulfur with a relatively slight increase
only in the ethereal sulfate sulfur fraction.   The dosage employed
by Lawrie is far beyond that used by others who have obtained
evidence of greater power of synthesis.   He was able to isolate

only 0.64 gm. of impure iodophenylmercapturic acid after inject-
ing 43.2 gm. of iodobenzene in daily doses of 3.6 gm. into rabbits.
This amount of the mercapturic acid is equivalent to 0.357 gm. of
iodobenzene or less than 0.1 per cent of the benzene derivative fed.
In sharp contrast to these results is the work of Nishimura (6), who
by feeding the monohalogen benzene derivatives in amounts of
0.5 to 1.0 gm. daily to well fed or fasting rabbits was able to isolate
the corresponding mercapturic acids in amounts ranging from 2.61
to 6.48 per cent of the benzene derivative fed.   The highest per-
centages of isolated material were obtained after the bromobenzene
feeding and the lowest after chlorobenzene.   We believe that our
results and those of Nishimura indicate that in small doses the
monohalogen derivatives are as effectively metabolized in the
organism of the rabbit as in the dog.

It may also be pointed out that the diet used in Lawrie's experi-
ment contained a relatively large amount of green food (150 gm. of
cabbage) and a relatively small amount of oats (20 gm.), a diet
which, according to the experiments of Abderhalden and Wert-
heimer (5), is unfavorable for the synthesis.   In our own feeding
experiments, the basal diet contained 70 gm. of oats and 100 gm. of
cabbage and was consumed completely by the animals except on 1
experimental day.

SUMMARY

After the oral administration of moderate amounts (0.4 to 0.7
gm. per kilo of body weight) of monobromobenzene to the rabbit,
the changes in the distribution of urinary sulfur (increased excre-
tion of ethereal sulfate and organic sulfur and decreased excretion
of inorganic sulfate sulfur) were comparable to those observed in
similar experiments with the dog.   It is believed that the reac-
tions by which the monohalogen derivatives are metabolized in
the organism of the rabbit are similar to those in the organism of
the dog.

BIBLIOGRAPHY

1. Hele, T. S., *Biochem. J.*, **18**, 586 (1924).
2. Coombs, H. I., and Hele, T. S., *Biochem. J.*, **21**, 611 (1927).
3. Yamaguchi, *Kyoto Igaku Zassi*, **26**, No. 5, cited by Nishimura (6).
4. Rhode, H., *Z. physiol. Chem.*, **124**, 15 (1922–23).

5. Abderhalden, E., and Wertheimer, E., *Arch. ges. Physiol.*, **207,** 215 (1925); **209,** 611 (1925).

6. Nishimura, K., *Acta schol. med. univ. imp. Kioto*, **12,** 73 (1929–30).

7. Lawrie, N. R., *Biochem. J.*, **25,** 1037 (1931).

8. White, A., and Lewis, H. B., unpublished data.

9. Fiske, C. H., *J. Biol. Chem.*, **47,** 59 (1921).

10. Baumann, E., and Preusse, C., *Ber. chem. Ges.*, **12,** 806 (1879).

11. Coombs, H. I., *Biochem. J.*, **21,** 623 (1927).

IRRADIATED MILK: THE ENERGY REQUIREMENTS FOR ANTIRACHITIC ACTIVATION

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The irradiation of milk is a complex problem from the physical, optical, and photochemical standpoint. The knowledge concerning the antirachitic activation of pure sterols with ultra-violet radiation cannot be applied directly to milk for several reasons. The provitamin or activatable substance as it exists in milk is associated with other materials in solution and suspension. Such substances may act as internal optical filters and absorb part of the radiations that might otherwise activate the provitamin. The provitamin as it exists in milk is possibly not a pure sterol; it may exist in a complex combination with lipoids and other compounds which might affect its photochemical sensitivity.

Laboratory and commercial experience has indicated that ultra-violet radiation which has been used for activation of pure sterols is useful for activating milk antirachitically. A review of the published data concerning milk irradiation shows that exposure periods in various laboratories have varied from a few seconds to as long as 2 hours (1–5). While the available data show that such milks have a measurable degree of antirachitic potency, the extreme variations in the period of exposure indicate an obvious lack of standardization in the technique of irradiating milk. The factors involved are the composition of the milk, the method of

application, and the source of the radiation.   The composition of the milk may be considered relatively constant, whereas the energy from the source of radiation may be subject to wide fluctuations.   This is particularly true of the type of lamps which have been used for almost all of the experiments recorded up to this time.   A recent analysis by Dingee (6) indicates that variations between individual mercury lamps are unpredictable and may be as great as 16-fold.   With such fluctuations existing in the source of radiation, variations in the results are to be expected.

In recent years physical researches have developed new sources of ultra-violet radiation, the qualities of which are constant and reproducible.   Likewise, methods of analysis of radiation have been developed so that constant or variable sources can be described with precision, both as to quality and quantity of radiation. This knowledge is now available and can be used in combination with photochemical and biological investigations pertaining to milk irradiation with promise of contributing to our knowledge of the nature and mechanism of the changes which occur.   Such developments, supplemented with clinical data, provide a further basis for the study of the synthesis of vitamin D and the rôle of this vitamin in human nutrition.

In 1924, Hess (7) and Steenbock (8) independently discovered that ultra-violet rays conferred antirachitic and calcifying properties on inert substances.   Subsequently, Hess and Weinstock (9) demonstrated that such properties were due to specific parts of the ultra-violet spectrum acting upon the sterols contained in the irradiated products.   The data reported by Fosbinder et al. (10), Kon and collaborators (11), Marshall and Knudson (12), and Maughan (13) dealing with the physical energy requirements involved in the preparation of activated sterols furnish practically the only evidence of such character now available.   Few or no data have been published showing the relationship of such principles to the preparation of activated food products.

Hess and Weinstock (1) indicated as early as 1925 the potential benefits of irradiated fluid or dry milk as a means of combating infantile rickets.   Numerous laboratory and clinical results both in this country and abroad have indicated the merits of irradiated milk when prepared under standardized and controlled conditions. The use of irradiated milk prepared on a large commercial scale has

been carried out mainly in Europe, especially by Scheer in Frankfort and Wieland in Basel. Supplee and coworkers (4, 5) have shown that milk is activated to a substantial degree by exposure for only a few seconds to ultra-violet radiations and that such milk underwent none of the adverse changes which are manifested by milk irradiated for longer periods of time. Obviously, activated milk intended for infant feeding or for other dietary purposes should be safeguarded from the deleterious qualities which become evident in overexposed or promiscuously irradiated products. The efficiency of the technique of irradiation, the prevention of undesirable secondary reactions including objectionable flavor and odor, and the uniformity of the potency imparted to the product necessitate a detailed knowledge of the character of the energy. In the commercial irradiation of milk, therefore, the precise nature of the radiant energy used as well as the practical coordination of facilities required for exposing large volumes to ultra-violet radiation for short periods of time must be taken into account. Standardized sources of the active rays in which the quality and quantity of the energy output are uniformly maintained, are therefore essential for the study of the various factors concerned in the irradiation of milk and for the production of a uniform product.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Sources and Character of Radiation*</div>

Since standardized sources of radiation are now available, the use of such facilities for the irradiation of large quantities of milk under controlled conditions seemed urgent in order that the biological assay of and the clinical results from the treated product might be coordinated with the physical data. The radiation used in these investigations was obtained from various types of carbon arcs and the mercury arcs in quartz.

The carbon arcs were of the flaming arc type recently developed for experimental and industrial application of ultra-violet rays (14, 15). The arcs were formed between electrodes of various compositions. The carbon arc is of such nature that under constant electrical conditions, namely constant current and voltage of the arc stream itself between electrodes of a given kind, a con-

stant amount and quality of radiation are emitted.   This radia-
tion is constant irrespective of variations of a transient nature,
such as altered room temperature, and also is free from any altera-
tion due to age of the lamp, since the electrodes when consumed
after several hours of arc operation are replaced with new ones.
During the life of the carbons the radiation is as constant as the
electrical conditions of the arc, which for these experiments were
automatically controlled by a motor-operated mechanism which
maintained approximately constant electrical conditions at the
arc even though the line voltage may have varied or other dis-
turbances occurred.   The amount of energy supplied to the carbon
arcs may be varied between wide limits by controlling the electrical
characteristics of the lamp circuit.   Accordingly, the quantity of
radiation from a given arc could be varied at will since the amount
of radiation emitted is a known function of the power consumed.
The kind of radiation could likewise be controlled by several
methods.   The chemical composition of the electrodes can be
altered by adding inorganic substances to the carbon, which
procedure produces wide variations in the character of the visible
and ultra-violet radiation (16).

   The problem of using the mercury arcs to secure known amounts
of radiation is more complex.   The age of the burner, varying
electrical conditions of the supply current, and the temperature
of the room are among the variables that may alter the intensity
of the irradiation by as much as 16:1 (16).   The mercury arc in
quartz is an electric arc in mercury vapor between mercury or
metal electrodes.   It is entirely enclosed in a quartz tube to
prevent loss of the mercury vapor and is subject to much the same
physical laws as any other type of arc.   It is, however, limited to a
few hundred watts in power consumption, whereas a carbon arc can
be varied from a few watts to several thousand watts with the
output increasing at a greater rate than the input.   Even if every
external and electrical condition is maintained in the operation
of the mercury arc there is still a variation from lamp to lamp and
a variation in the same lamp with the increasing age of the burner
caused by changing transparency of the quartz tube enclosing the
arc.   Therefore, even though the intensity of radiation generated
by the arc stream is constant, due to constant electrical conditions,
the radiation emitted by the lamp will change if the transparency

of the quartz tube changes since the radiation must pass through the walls of this tube. Some of these tubes lose their power of transmission but slowly, others deteriorate rather rapidly. It is impossible to predict the behavior of any particular burner.

In this investigation the amount of radiation from the various burners was measured and controlled by appropriate means. During the operation of the mercury arcs an approximately constant room temperature was maintained and a voltage regulator
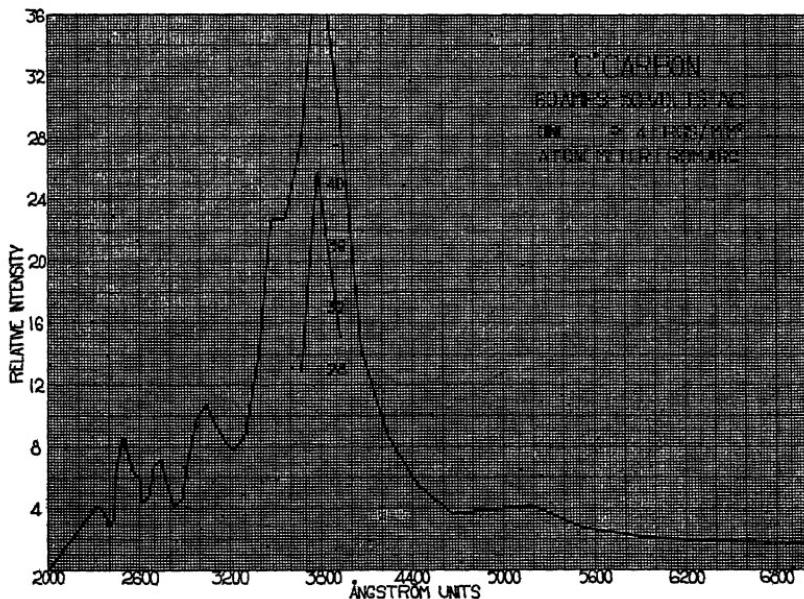
CHART 1. Spectral energy distribution curve for C carbon; 60 amperes, 50 volts. Each square represents 4 ergs per sq. mm. at 1 meter from the arc.

was installed in the electrical supply lines to furnish constant voltage.

The amount of radiation from the mercury arcs was determined by making spectroradiographic analyses of some of the typical burners used in the assembly. These analyses were made immediately following the irradiation of the milk. During the actual period of irradiation the constancy of the energy output was determined by the use of a Rentschler meter (17). The electrical

characteristics were then duplicated in the physical laboratory for the purpose of reproducing for physical analyses radiations of the same character as were used during the actual treatment of the milk.   The reproduction of the same conditions was again checked by the Rentschler meter and found to be the same as was actually used during the irradiation of the milk.   The Rentschler meter measures ultra-violet radiation from 2500 to 3400 Å.
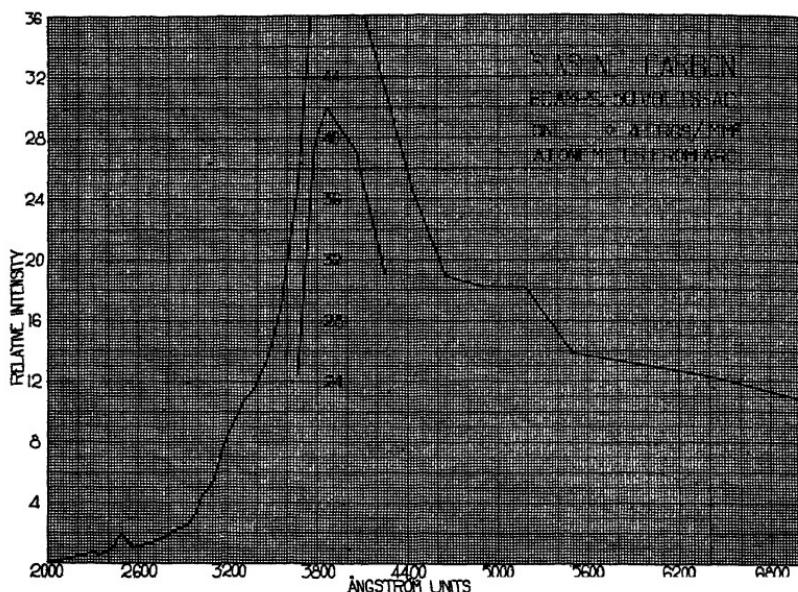
CHART 2.   Spectral energy distribution curve for sunshine carbon; 60 amperes, 50 volts.   Each square represents 4 ergs per sq. mm. at 1 meter from the arc.

The energy output from the various carbon arcs had been determined previously by spectroradiographic analyses under various known electrical conditions.   For these experiments various electrical conditions from which definite data were available were reproduced in the operation of the carbon arcs during the irradiation of the milk.   Since the character of the radiation from the carbon arcs is readily reproducible under the same electrical conditions as heretofore noted, it may be assumed that the results from the spectroradiographic analysis as shown hereinafter were

applied during the treatment of the milk used in these experiments.

The radiation from the various sources is described by Charts 1 to 4 and in Table I. The graphs show the relative quantities of
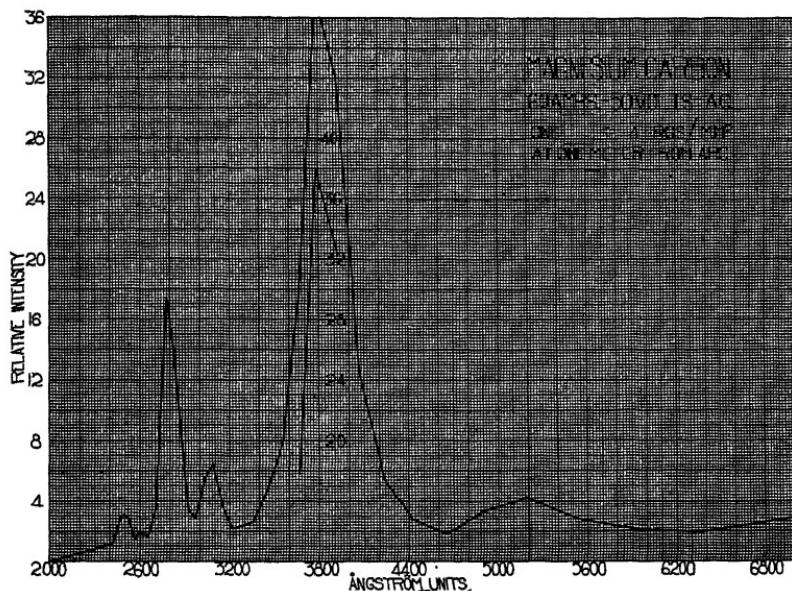
CHART 3. Spectral energy distribution curve for magnesium carbon; 60 amperes, 50 volts. Each square represents 4 ergs per sq. mm. at 1 meter from the arc.
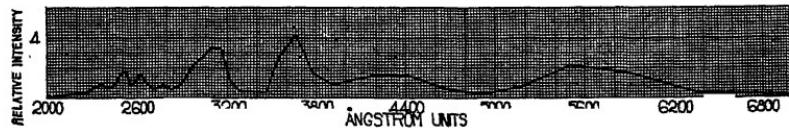
CHART 4. Spectral energy distribution curve for mercury vapor arc after 850 burning hours; 3.7 amperes, 160 volts. Each square represents 4 ergs per sq. mm. at 1 meter from the arc.

the different kinds of radiation through the region of the visible and ultra-violet.

The physical data were obtained by the aid of a spectroradiometer by the established methods (14–16). The procedure is to disperse a beam of the radiation into a spectrum by a quartz lens

TA¹

Character of Radiation between 2000 and 3400 Å. Used for Irradiating Milk

Group No.	Sample No.	Arc	Electrical characteristics		Ergs per sec. per sq. mm. at normal incidence to milk surface 24 in. from arc							
			amp.	volts	2000–2200Å.	2200–2400Å.	2400–2600Å.	2600–2800Å.	2800–3000Å.	3000–3200Å.	3200–3400Å.	Total 2000–3400Å.
I-AC-60	5– 8	C carbon	60	50	7.56	19.44	34.56	31.32	35.64	50.22	50.76	229.50
I-AC-80	9– 12	" "	80	50	12.98	33.48	55.08	45.36	48.60	72.90	72.36	340.74
II-AS-60	65– 68	Sunshine carbon	60	50	0.76	3.24	7.56	8.10	14.04	30.24	56.16	120.10
II-AS-80	69– 72	" "	80	50	0.97	4.32	10.80	10.80	17.28	37.80	75.60	157.57
III-AMG-60	125–128	Magnesium carbon	60	50	1.66	4.44	11.95	38.40	37.00	26.60	13.90	134.00
III-AMG-80	129–132	"	80	50	2.78	7.43	19.60	60.00	59.90	38.90	19.03	207.64
IV-AHG	181–184	Mercury vapor*	3.7	160	4.43	10.65	24.10	19.60	27.50	65.30	12.88	164.44

he   itead of 24 inch

and a prism, and to measure the relative amounts of the radiation in narrow adjacent spectral bands by a thermopile-galvanometer system.   This procedure determines the shape of the curve.   The instruments and the total radiation are calibrated against a standard of radiation and this determines the height of the curve. From such curves the relative energy of different regions of the spectrum can be determined by measuring the area under the curves in the region in question.   Data such as those shown in Table I can then be prepared showing the number of ergs per second of energy of given wave-lengths per sq. mm. of area at a given distance from the arc.   With these data, showing the kind and amount of energy, a study of the effect of radiation on milk is analogous to the addition to the milk of a measured amount of a reagent of known purity.

Irradiation of Milk with Carbon Arcs and Mercury Arcs

The carbon arcs and mercury arcs operated under known conditions and with known energy output, as shown in the accompanying graphs (Charts 1 to 4) and Table I, were used for the irradiation of milk, with the equipment of a commercial milk-handling establishment.   The accrued experiences of the past 5 years at this particular plant during the production of irradiated dry milk, in conjunction with the accurately determined and controlled energy of the different light sources, provided a most suitable arrangement for the study of the technique of irradiating milk with laboratory precision under actual conditions of commercial production.

For this work, fluid milk containing 1.2 per cent of butter fat was used.   The experiments were made during a period of 30 consecutive days during the autumn.   Milk from the same territory and general source of supply was used throughout.   The amount of milk irradiated under any one particular set of conditions was approximately 1000 pounds.   The milk was exposed to the radiation in the form of a moving film which received the rays at constantly changing angles of incidence varying from 0 to 90 degrees.   The complete cycle of exposure to the range of impingement angles required a period of about 0.3 second.   The thickness of the milk film was maintained at substantially 0.4 mm.   The periods of exposure after which observations were made were, as a

rule, 8, 16, 32, and 48 seconds.   Immediately following irradiation, the fluid milk was dried by the Just process and the desiccated product hermetically sealed in inert gas.

Since it was desired to study the different effects resulting from the qualitative and quantitative control of the radiant energy, various screens were used.   These were supplemented by further variables in the experimental conditions, for example, the effect of such factors as direct and reflected light, reflecting material, design of reflector, different arc currents, and distance of light source from the milk film.   During the course of this work 1000 pound lots of fluid milk were irradiated under 238 different conditions, each involving an exposure to different radiations, and each readily subject to duplication.   Although the objective of this work was a comprehensive investigation of the properties of milk irradiated in various ways, only the antirachitic and calcifying potency of milk exposed to unscreened radiations will be reported at this time.

Antirachitic and Calcifying Properties of Irradiated Milk

The biological assay of the experimental samples was carried out according to established procedures.   White rats reared on a standardized ration were selected from our stock colony at 28 to 30 days of age.   During the test period they were maintained in individual metal cages with screen bottoms.   The Steenbock and Black (18) rachitogenic Ration 2965 was fed for 21 days followed by 10 days of supplemental feeding of 10 cc. daily of the reconstituted dry milk.   At the end of this period the line test and bone ash determinations were made.   The results as shown in Table II are the averages obtained for groups of not less than four animals.

These data demonstrate a definite correlation between the quality and quantity of the radiant energy applied to milk and the resulting antirachitic and calcifying potency.   The relative merits of the mercury arc and various carbon arcs unscreened are also indicated by these results.   The biological assays based on the 10 cc. feeding level were further substantiated by the results from feeding at 2 and 6 cc. levels.   These results are considered as confirmatory in showing that substantially the maximum antirachitic potency which could be imparted to milk was brought

TABLE II

Antirachitic and Calcifying Potency of Milk Irradiated with Definite Amounts of Energy (2000–3400 Å.) from Various Sources

Group No.	Sample No.	Arc	Exposure period	Total ergs applied per cc. milk, (2000-3400 Å.)	Bone ash	Rickets healing (average line test)
			sec.		*per cent*	
I-AC-60	5	C carbon	8	1,790,000	32.76	1.6
	6		16	3,654,000	38.66	3.8
	7		32	7,751,000	36.06	3.2
	8		48	12,276,000	37.14	3.4
I-AC-80	9	" "	8	2,302,000	32.25	2.6
	10		16	5,026,000	36.82	3.5
	11		32	10,158,000	38.86	3.4
	12		48	16,790,000	38.06	3.6
II-AS-60	65	Sunshine carbon	8	911,000	31.76	1.4
	66		16	1,910,000	31.90	1.8
	67	.	32	3,675,000	30.96	2.9
	68		48	5,628,000	32.17	2.5
II-AS-80	69	" "	8	1,247,000	34.22	2.8
	70		16	2,491,000	36.78	2.2
	71		32	5,084,000	34.50	3.0
	72		48	7,597,000	35.07	3.4
III-AMG-60	125	Magnesium carbon	8	1,158,000	31.60	2.9
	126		16	2,250,000	37.58	2.5
	127		32	4,543,000	38.68	3.5
	128		48	6,895,000	36.17	3.2
III-AMG-80	129	" "	8	1,692,000	32.14	3.6
	130		16	3,358,000	36.78	3.6
	131		32	6,551,000	39.28	4.0
	132		48	10,576,000	38.60	3.8
IV-AHG	181	Mercury vapor	8	651,000	31.26	1.4
	182		16	1,235,000	33.79	2.4
	183		32	2,529,000	33.14	3.3
	184		48	3,685,000	34.78	3.4
	238	Non-irradiated	0	None	31.06	No healing

about within the first few seconds of exposure when a suitable quality and quantity of radiant energy was used. It is to be

TABLE III

Prevention of Infantile Rickets by Irradiated Milk of Low Fat Content Given as Reconstituted Dry Milk

Name	Date	Age	Weight	Beading	Craniotabes	Bowing	Date	Result	Remarks
				Physical examination			Roentgenogram		
		mos.	lbs.						
V.C.	Feb. 9	2½	10¾	+	−	+	Mar. 19	Normal	Negro
	Mar. 18	3⅓	12⅙	+	−	+			
M.P.	Feb. 9	1	7	−	−	−	" 24	"	"
	Mar. 18	2⅓	9	−	−	±			
C.P.	Feb. 9	6	11¾	±*	−	±	" 24	"	"
	Mar. 18	7½	14⅛	+*	−	+			
E.C.	Feb. 9	2½	10	±	+	+	" 31	"	"
	Apr. 4	4½	13	±	+	++			
M.R.	Feb. 9	1½	6	−	−	−	Apr. 2	"	"
	Mar. 18	3	8½	±	−	+			
E.P.	Feb. 9	4	10¼	±*	−	+	Mar. 24	"	"
	Apr. 4	6	12¾	+*	−	+			
R.T.	Feb. 9	2	10⅛	−	−	±	" 31	"	"
	Apr. 4	4	13¾	±	−	+±			
F.G.	Feb. 9	3	12¼	−	+	+	" 24	Marked calcification	"
	Mar. 28	4⅔	14½	±	−	+±			
L.R.	Feb. 9	3	9	±	++	+	" 24	"	"
	Mar. 18	4½	10¾	±	+±	+			
M.H.	Feb. 9	2½	11¼	±	−	±	" 24	"	"
	Mar. 25	4	14	±	−	+			
J.M.	Feb. 9	3	13½	±	++	+	" 24	"	"
	Mar. 18	4⅓	16⅝	±	++	+			"
									gained 50 oz. in 37 days

* Angular beading.

noted that the objectionable flavor and odor frequently reported as developing in milk after exposure to ultra-violet rays was not

detectable within the period required to obtain the maximum or substantially maximum antirachitic potency which could be imparted to the milk irradiated under the conditions of these experiments, provided sufficient energy within the antirachitic range (below 3150 Å.) was available.

Clinical Results

An opportunity was afforded to make a clinical test of some of the milk which has been irradiated under controlled conditions by the carbon arc rays. Although the test was not carried out on a large scale, its results conclusively demonstrated the antirachitic potency of this product. Table III shows that the series of infants which were given this milk during the height of the winter were fully protected from rickets; indeed, marked calcification of the epiphyses was evident during the month or more which they were given the preparation which is designated in Table II as Sample 5. Carbon "C" was used for activating this lot of milk, and a total of 3,654,000 ergs between 2000 and 3400 Å. was applied per cc. of milk during a period of 16 seconds under conditions previously described. We must take into consideration that the group was composed of colored infants, which rendered the test particularly severe. The observations were carried out by Dr. J. M. Lewis in one of the health clinics of the Department of Health of New York City.

It should be added that this milk was found to be also of curative value in a number of cases. In the course of 1 or 2 months it brought about definite or marked healing as manifested by Roentgenograms. These tests were likewise carried out during the months of February and March on colored children. We do not wish to emphasize the curative value of this irradiated milk, but rather, that milk treated in this way is of definite value in protecting infants from rickets.

SUMMARY

1. Physical data indicating the character and the amount of radiant energy required for the antirachitic activation of milk are presented and coordinated with the biological and clinical tests. The irradiation of the milk was carried out in 1000 pound lots under commercial conditions which permitted a precise study

2. With a suitable quality and quantity of radiant energy applied under uniform conditions, the antirachitic and calcifying properties of milk can be regularly increased within a few seconds, as determined by biological laboratory assays and clinical tests on infants. This high degree of activation is obtained without the development of adverse secondary reactions which may occur after longer periods of exposure.

3. The energy of the radiations from a suitable carbon lamp of the flaming arc type and a quartz mercury vapor lamp confers definite antirachitic and calcifying potency on milk treated according to the technique described in this paper. However, the spectral energy characteristics of the radiations from the carbon arcs are readily subject to control, both as to the quality and quantity of the energy.

4. Milk irradiated under controlled conditions described in this paper and subsequently dried by the Just process was found to protect even colored infants from rickets during the winter months. Such milk manifested definite curative value in a number of cases, as shown by the marked healing in Roentgenograms.

BIBLIOGRAPHY

1. Hess, A. F., and Weinstock, M., *J. Biol. Chem.*, **64**, 181 (1925).
2. Kramer, B., *Am. J. Dis. Child.*, **30**, 195 (1925).
3. Steenbock, H., Hart, E. B., Hoppert, C. A., and Black, A., *J. Biol. Chem.*, **66**, 441 (1925).
4. Supplee, G. C., and Dow, O. D., *Am. J. Dis. Child.*, **34**, 364 (1927).
5. Supplee, G. C., Flanigan, G. E., Kahlenberg, O. J., and Hess, A. F., *J. Biol. Chem.*, **91**, 773 (1931).
6. Dingee, A. L. M., *J. Physic. Therapy*, **48**, 229 (1930).
7. Hess, A. F., *Am. J. Dis. Child.*, **28**, 517 (1924).
8. Steenbock, H., and Black, A., *J. Biol. Chem.*, **61**, 405 (1924).
9. Hess, A. F., and Weinstock, M., *Proc. Soc. Exp. Biol. and Med.*, **24**, 759 (1926–27).

10. Fosbinder, R. J., Daniels, F., and Steenbock, H., *J. Am. Chem. Soc.*, **50**, 923 (1928).
11. Kon, S.-K., Daniels, F., and Steenbock, H., *J. Am. Chem. Soc.*, **50**, 2573 (1928).
12. Marshall, A. L., and Knudson, A., *J. Am. Chem. Soc.*, **52**, 2304 (1930).
13. Maughan, G. H., *Am. J. Physiol.*, **85**, 392 (1928).
14. Dorcas, M. J., *Ind. and Eng. Chem.*, **22**, 1244 (1930).
15. Greider, C. E., *Ind. and Eng. Chem.*, **23**, 508 (1931).
16. Coblentz, W. W., Dorcas, M. J., and Hughes, C. W., *Bureau Standards, Sc. Paper 539* (1926).
17. Rentschler, H. C., *J. Am. Inst. Elec. Eng.*, **49**, 113 (1930).
18. Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).

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BIBLIOGRAPHY

1. Hess, A. F., and Weinstock, M., *J. Biol. Chem.*, **64**, 181 (1925).
2. Kramer, B., *Am. J. Dis. Child.*, **30**, 195 (1925).
3. Steenbock, H., Hart, E. B., Hoppert, C. A., and Black, A., *J. Biol. Chem.*, **66**, 441 (1925).
4. Supplee, G. C., and Dow, O. D., *Am. J. Dis. Child.*, **34**, 364 (1927).
5. Supplee, G. C., Flanigan, G. E., Kahlenberg, O. J., and Hess, A. F., *J. Biol. Chem.*, **91**, 773 (1931).
6. Dingee, A. L. M., *J. Physic. Therapy*, **48**, 229 (1930).
7. Hess, A. F., *Am. J. Dis. Child.*, **28**, 517 (1924).
8. Steenbock, H., and Black, A., *J. Biol. Chem.*, **61**, 405 (1924).
9. Hess, A. F., and Weinstock, M., *Proc. Soc. Exp. Biol. and Med.*, **24**, 759 (1926–27).

10. Fosbinder, R. J., Daniels, F., and Steenbock, H., *J. Am. Chem. Soc.*, **50**, 923 (1928).
11. Kon, S.-K., Daniels, F., and Steenbock, H., *J. Am. Chem. Soc.*, **50**, 2573 (1928).
12. Marshall, A. L., and Knudson, A., *J. Am. Chem. Soc.*, **52**, 2304 (1930).
13. Maughan, G. H., *Am. J. Physiol.*, **85**, 392 (1928).
14. Dorcas, M. J., *Ind. and Eng. Chem.*, **22**, 1244 (1930).
15. Greider, C. E., *Ind. and Eng. Chem.*, **23**, 508 (1931).
16. Coblentz, W. W., Dorcas, M. J., and Hughes, C. W., *Bureau Standards, Sc. Paper 539* (1926).
17. Rentschler, H. C., *J. Am. Inst. Elec. Eng.*, **49**, 113 (1930).
18. Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).

THE ANTINEURITIC VITAMIN

I. THE METHOD OF ASSAY, CONCENTRATION OF THE VITAMIN WITH SILVER UNDER VARIOUS CONDITIONS, AND ITS SOLUBILITY IN CERTAIN ORGANIC SOLVENTS*

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I. *Method of Assay Used in These Investigations*

In an attempt to repeat the study of Jansen and Donath (1927) on the isolation of antineuritic vitamin B we have made several observations of interest. Satisfactory description of this work necessarily involves the question as to the adequacy of the biological test employed in assaying the many fractions obtained. Inasmuch as there is no unanimity concerning the proper method to use when testing for antineuritic vitamin potency, it is necessary first of all to describe the particular technique which we have used in these investigations.

Plan

The assay in these studies is performed by a combination of weight-maintenance and curative techniques carried out on pigeons subsisting on the dietary régime below. The plan differs from that of Seidell (1917, 1922), Funk and Paton (1922), Kinnersley and Peters (1925), Williams and Waterman (1928), and others in one very important respect; namely, in that polished rice does not constitute the *sole* diet. It has been known for a considerable period that polished rice is not a complete food; that it is deficient not only with respect to antineuritic vitamin B, but protein, mineral nutrients, and fat-soluble vitamins as well. In view of

this fact it appears logical for the investigator in this field who wishes to use polished rice in his diet to supplement it as well as possible so as to supply a food mixture deficient only with respect to the one variable of interest; namely, antineuritic vitamin B. This we have attempted to do. Our approach to the problem therefore differs from that of the workers mentioned above because these investigators experimented with polished rice supplemented simply by various vitamin B extracts in an endeavor to secure maintenance nutrition in the pigeon. Even Carter, Kinnersley, and Peters (1930, a) in a very recent study fed polished rice supplemented only with such products as cod liver oil and various extracts containing antineuritic vitamin. They concluded that antineuritic vitamin B alone as the supplement for polished rice does not afford maintenance. This may very well be the case. It is important in this connection to note, however, that Jansen and Donath (1926) found that their rice vitamin preparation did not effect weight maintenance in pigeons fed polished rice unless fed along with another substance (or substances?) which could be furnished in their experience by meat powder thoroughly extracted with boiling water. From this it appears obvious that the weight-maintenance technique with the pigeon may be of service in the assay for antineuritic vitamin, provided the birds also receive meat residue, cod liver oil, and a suitable salt mixture as part of their basal ration.

We believe that the inadequacies of a sole diet of polished rice are also revealed by the behavior of birds restricted to this ration for more than about a month. Carter, Kinnersley, and Peters (1930, a) comment on this fact. When, therefore, investigators feed birds for approximately 2 months on polished rice supplemented only by the products under assay, it is not surprising that the birds, although being relieved of their polyneuritic symptoms, fail to regain lost weight, an observation suggesting the existence of new hitherto unappreciated dietary essentials, as Williams and Waterman (1928) report.

The weight-maintenance technique used in the investigation reported in this paper is an extension of earlier studies conducted in this laboratory by Klotz (1926). The appearance of the subtle anorexia for a vitamin B-deficient diet is taken as the first sign that the organism's need for antineuritic vitamin is not being

met. This characteristic loss of the urge to eat is considered to be present in the pigeon subsisting on the dietary régime employed in this assay when the bird fails to ingest voluntarily enough calories to maintain normal weight. The bird is weighed daily except Sunday, and when a consistent steady decline in weight is noted over a period of at least 5 days, it is concluded that the characteristic anorexia has supervened, so that the bird is a proper subject upon which to test a preparation for content of antineuritic vitamin B. Recent researches have shown that the heat-stable vitamin G (or B_2) factor in the old undifferentiated vitamin B plays no rôle in maintenance of the urge to eat or the development of this subtle anorexia (Cowgill, Rosenberg, and Rogoff, 1931, *a*; Burack and Cowgill, 1931; Sherman and Sandels, 1931). The antineuritic vitamin B is undoubtedly the chief, if not the sole agent involved here. We conclude, therefore, that our technique determines the presence of the antineuritic component of the vitamin B complex.

Many workers, notably Kinnersley and Peters (1928), have preferred the curative test when assaying materials for antineuritic vitamin. On the basis of our experience with different species of animals it is difficult to decide when the polyneuritic condition in a given animal is of a severity equal to that of a companion individual. Our objections to the pigeon curative technique as the sole method of assay are essentially those summarized by Smith (1930) and therefore need not be repeated here. We have gained the impression that the curative method, although valuable because of its objective character, is not as well suited as a weight-maintenance technique for detection of moderate or slight differences in potencies of vitamin preparations, such as an investigator desires when endeavoring to isolate the antineuritic factor. This is supported by the report of curative tests of the Jansen-Donath vitamin crystals with the Kinnersley and Peters technique, concerning which Jansen, Kinnersley, Peters, and Reader (1930) remarked, "It is to be noted that there is a rather considerable variation in the pigeon tests, all of which have been here included. The variation is larger than that to which we are accustomed with yeast preparations." Even with these objections in mind there can be no doubt as to the advantage of an *objective* demonstration of antineuritic vitamin potency, such as a cure represents, over

any indirect method. Therefore, in our assay, tests by the weight-maintenance method are supplemented by trials on polyneuritic birds for curative power, and the latter regarded as confirmatory, and quantitative only in a rough way.

At least three pigeons are used for each assay. The usual procedure has been to determine by preliminary trials on one or more birds the approximate daily dose required and then to confirm this and determine the required dose more accurately on at least two other birds. The larger changes in dose made in an endeavor to approximate the daily minimum are thus made on one pigeon, and slight changes on the confirmatory birds. This results in considerable saving of time when numerous fractions are at hand to be tested. The results are expressed in terms of the *pigeon unit,* which may be defined as *the amount required to maintain the body weight constant over a period of from 10 to 14 days in the case of a 300 gm. pigeon fed according to the method described in this paper.* When the weight of a given bird differs markedly from 300 gm. the measured dose is corrected by a calculation based on the relationship between body weight and vitamin minimum discovered by Cowgill and Klotz (1927); namely, vitamin $= K$ weight$^{\frac{2}{3}}$, (see Table II) in which K is merely an equating constant. The agreement of assays on several birds of approximately 300 gm. of body weight with those for larger or smaller pigeons *after correcting for the difference in size* has proved to be good, and has served further to strengthen our conviction concerning the validity of the relationship stated above. The assays are reported in terms both of mg. of solids and mg. of nitrogen per pigeon unit.

Care of Birds

The pigeons are housed in small cages which necessarily restrict movement somewhat and thus reduce the metabolism occasioned by exercise to a minimum fairly constant amount. The work of Cowgill, Rosenberg, and Rogoff (1931, b) on the effect of exercise on the vitamin B requirement may be cited as evidence of the importance of reducing the exercise factor to a minimum. For this reason the practice of caging numerous birds in the same pen, where considerable flying is possible, should be avoided. When cages about 18 inches long, 10 inches wide, and 12 inches deep are used, there appears to be no disadvantage in housing

two birds together. Cages suitable for metabolism work with rabbits have proved to be serviceable, because the solid wall rising about 6 inches from the bottom of the cage serves to confine within the cage any rice scattered by the birds. Each cage is provided with containers for water, polished rice, and stone grit.

Diet

Polished rice is offered *ad libitum* and is intended to serve as the chief source of calories. The birds also receive daily a forced administration of commercial meat residue[1] as a source of good protein and heat-stable vitamin G (B$_2$) factor relatively free from antineuritic vitamin (Osborne and Mendel, 1917; Cowgill, 1926–27), the Osborne-Mendel salt mixture (1917) designed to furnish mineral nutrients, and cod liver oil, intended as a source of fat-soluble vitamins. These supplements are placed in a No. OOO gelatin capsule and one capsule is given daily to each bird. This feeding technique is very similar to that used by Jansen and Donath, a fact that we learned after our first experiments (Cowgill and Klotz, 1927) had been completed. In our method, however, inorganic nutrients also are supplied.

Inasmuch as the administration of these supplements is designed to meet the deficiencies of the polished rice, it is pertinent to consider whether the quantities of the respective supplements contained in each capsule are sufficient. About 7 per cent of polished rice is protein.[2] From the weights of numerous filled capsules and the nitrogen content of the meat residue it is estimated that each capsule furnished about 0.9 gm. of meat-residue protein. It is assumed that the birds will adjust their energy intake according to their energy demands (Cowgill, 1928). Therefore pigeons ranging from 250 to 600 gm. of body weight will consume from 52 to 93 calories per day, or from 14 to 25 gm. of rice, and receive from 1 to 1.8 gm. of rice protein per day. Therefore the total amount of protein ingested daily will range from 1.9 to 2.7 gm. On this basis the protein calories constitute from 15 to 12 per cent of the total, the higher figure corresponding to the smaller birds which are the ones most commonly used. Students of nutrition are aware that this level of protein intake is quite sufficient. From

[1] From the Valentine Meat Juice Company, Richmond, Virginia.

[2] Rosenheim, O., and Kajiura, S., *J. Physiol.*, **36**, p. liv (1908).

this it is evident that our feeding technique guards against any loss of body weight being due to shortage of protein.

The requirement for inorganic nutrients was met by means of the Osborne-Mendel salt mixture. This material filled the concavity of the end of the capsule and weighed on an average from 0.075 to 0.1 gm. Fat-soluble vitamins A and D were supplied daily by 2 drops of tested cod liver oil administered either directly to the bird by medicine dropper or contained in the capsule along with the other supplements.

The question arises as to whether this scheme of feeding prevents any possible weight loss by the pigeon due to lack of the heat-stable vitamin G (B$_2$) factor. Inasmuch as both muscle (Hoagland and Snider, 1930; Goldberger, Wheeler, Lillie, and Rogers, 1926) and a meat-residue preparation even more highly extracted than the product used in our experiments (Vars, 1931) have been found to contain fair amounts of this heat-stable substance, it must be concluded that this method of feeding supplies some vitamin G (B$_2$). At the present time there does not appear to be any satisfactory method by which one may calculate the vitamin G (B$_2$) requirement in order to determine whether the amount of meat residue given our birds daily supplies adequate amounts of this factor. Another pertinent fact is the recent finding (Cowgill, Rosenberg, and Rogoff, 1931, a; Burack and Cowgill, 1931; Sherman and Sandels, 1931) that vitamin G (B$_2$) does not play a rôle in the development of the anorexia characteristic of lack of undifferentiated vitamin B. In view of these considerations we feel justified in concluding that loss of weight by adult pigeons fed according to the technique described in this paper cannot be attributed to shortage of heat-stable vitamin G (B$_2$).

Williams and Waterman (1928) and, more recently, Eddy, Gurin, and Keresztesy (1930) have brought forward evidence interpreted to mean that the bird requires a third hitherto unrecognized factor believed to be present in undifferentiated vitamin B. According to the nomenclature favored by the English workers, this factor has been called vitamin B$_3$. Jansen and Donath (1927) who, like ourselves, gave their birds polished rice supplemented with meat residue and cod liver oil, obtained no evidence that this hypothetical third factor was lacking. Williams and Waterman explain this failure to confirm their finding by the assumption

that the new factor is present in either the meat residue or the cod liver oil. The same explanation might be offered for our results. Another reason may be cited for our view that, if birds require this hypothetical substance, it must be present in our basal diet. We have successfully fed pigeons on our basal diet supplemented by a concentrate of antineuritic vitamin for from 60 to 100 days with remarkable constancy of body weight during the entire period. The amount of solids furnished daily by the vitamin B_1 concentrate used in these particular instances was of the order of about 1.5 mg. Furthermore, the preparation of this concentrate involved procedures which, according to Eddy, Gurin, and Keresztesy, should have destroyed or inactivated the new vitamin B_3 factor. Evidently, then, the basal diet was supplying this needed nutrient.

Concerning the suggestion (Peters, 1929, 1930) that pigeons may also require for maintenance Reader's (1929, 1930) B_4 factor, or the more recently discovered alleged factor B_5 (Carter, Kinnersley, and Peters, 1930, b) we have no evidence from our own work to offer. We can only cite again the investigations of Jansen and Donath, who secured maintenance and protection against polyneuritis by feeding a basal diet of polished rice, meat residue, and cod liver oil, supplemented by very minute amounts of their crystalline vitamin preparation. Evidently these additional factors are contained in the meat residue or the cod liver oil.

Illustration of Method

In Table I are shown the actual assay data obtained with three pigeons tested with Concentrate I-A. It will be noticed that a curative test was performed with Pigeon 86. The data for body weight and measured dose shown in Table II were taken from Table I. The figures in the last column of Table II are doses corrected for a 300 gm. pigeon by the Cowgill-Klotz (1927) formula. In the case of the tests with Concentrate I-A it will be noticed that all three of the birds weighed more that 300 gm. In the case of the pigeons used in the assay of Concentrate I-B-4, two of the birds weighed less than 300 gm., and the actual assays, treated without regard to the weights of the individual birds, showed a variation of 14 per cent of the mean; when corrected to apply to the 300 gm. pigeon, the doses showed a variation of only 8 per

TABLE I

Assay Data for Antineuritic Vitamin B Concentrate I-A

Pigeon 89		Pigeon 86		Pigeon 83	
Dose	Daily body weight	Dose	Daily body weight	Dose	Daily body weight
cc.	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*
0	311	3.0*	238	0	328
	310				327
	Sunday	2.0	251		Sunday
	296				319
	287	1.0	260		318
					311
0.5	(287)†	0.5	272		
	289		286	0.2	(311)†
	298		308		309
	301		Sunday		316
	313		310		322
	Sunday		319		Sunday
	314		320		
			317	0.1	(Sunday)
0.2	(314)†				318
	322	0.2	(317)†		317
	324		323		313
	319		Sunday		320
	323		327		322
	328		336		317
	Sunday		330		Sunday
			326		320
0.1	(Sunday)		343		328
	324		344		318
	327		Sunday		
	327			0	(318)†
	327	0.1	(Sunday)		319
	—		344		309
	—		347		302
	Sunday		343		Sunday
	327		347		298
	338		350		

* Dose administered to cure neuritic symptoms.

† Each weight given is that measured on the *day following* the administration of a given dose; if the dose had any effect, one would expect to notice this on the following day rather than the same day. The weight enclosed in parentheses is that for the *same day* the new dose was begun; this weight was taken just before administering the test material.

TABLE I—*Concluded*

Pigeon 89		Pigeon 86		Pigeon 83	
Dose	Daily body weight	Dose	Daily body weight	Dose	Daily body weight
cc.	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*
0	(338)†		—		
	315		Sunday		
	313		337		
	309		342		
	305				
	Sunday	0	(342)†		
			332		
			332		
			323		
			325		
			Sunday		
			311		
			304		
			297		

TABLE II

Assay Data for Antineuritic Vitamin B Corrected to Apply to a 300 Gm. Pigeon

Test product	Pigeon No.	Average body weight over test period	Minimum dose	
			Measured	Corrected for 300 gm. pigeon
		gm.	*cc.*	*cc.*
Concentrate I-A	89	328	0.1	0.086
	86	344	0.1	0.080
	83	319	0.1	0.090
Average..				0.085
Concentrate I-B-4	89	281	1.2	1.34
	51	300	1.4	1.40
	63	277	1.0	1.60
	86	299	1.5	1.50
Mean....................................			1.27	1.46
Average deviation......................			0.18	0.12
" " in *per cent* of mean....			14	8.2
Corrected dose taken for comparison with results of assays of other preparations...........................				1.5

cent of the mean. These results are typical of many that might be cited.

II. Concentration of Antineuritic Vitamin by Silver under Various Conditions

Jansen and Donath (1927) have renewed interest in the chemistry of the antineuritic vitamin by the report of the isolation of a potent crystalline substance. This material prevented the appearance of polyneuritis in bondols (rice birds) in amounts of from 2 to 4 micrograms per day. The precipitation of the active material with little loss of potency was reported to take place between pH 4.5 and 6.5 in the presence of silver nitrate and barium hydroxide. In an attempt to repeat this work, we observed a considerable loss of activity at this point. Therefore, it was hoped that a study of the conditions of precipitation by silver and alkali would be useful. Various silver salts have been employed as a precipitant for the vitamin by many workers, but in so far as we are aware, there has been no estimation of the comparative efficiency of various combinations of acids, silver salts, and bases by one group of workers.

Funk (1911) reported that the antineuritic vitamin from rice polishings was precipitated by barium hydroxide in the presence of silver nitrate. The same author (1912–13) found that the vitamin extracted from yeast was not precipitated by silver nitrate-barium hydroxide unless the yeast had been previously hydrolyzed. An active fraction from wheat bran was obtained by Sullivan and Voegtlin (1916) by precipitating with silver acetate and barium hydroxide. Yeast vitamin was precipitated by the same reagents by bringing the reaction to pH 8 to 9 (Myers and Voegtlin, 1920). Kinnersley and Peters (1925) obtained a vitamin concentrate from yeast by adsorption on and elution from norit followed by an extensive alcoholic fractionation. The active substance in this concentrate was not precipitated by silver sulfate and sulfuric acid but was by silver nitrate and ammonia. A potent concentrate from rice polishings and yeast is obtained by treatment with silver picrate (Funk, 1927). Guha and Drummond (1929) found that if a vitamin concentrate from wheat germ is treated with silver oxide at pH 7 or by silver nitrate and barium hydroxide at pH 4.5 to 6.5, the vitamin is precipitated. Silver nitrate-barium

hydroxide between the pH values of 4.5 and 6.5 is a good precipitant for the antineuritic substance from rice polishings but not for that obtained from yeast (Williams, Waterman, and Gurin, 1930).

The plan of this research was to acidify the vitamin solution to pH 4, add an excess of a soluble silver salt, and remove the resulting precipitate. This precipitate, as was expected, contained little or no activity and was discarded. The filtrate was then brought to about pH 7.0 by the careful addition of a strong base, and the precipitate which formed at this point was removed. This second precipitate, after removal of the silver, was tested quantitatively for the antineuritic vitamin by the pigeon technique described in Part I of this paper. The assays were conducted as a separate investigation by one of us (G. R. C.) who was not informed concerning the details of the preparation of the products submitted for test.

In such an experiment there are three major variables, the acid, the silver salt, and the base. Sulfuric, nitric, or lactic acid was used as the source of hydrogen ions; barium or sodium hydroxide was employed to bring the reaction from pH 4 to pH 7.0; and to introduce silver we made use of silver lactate or nitrate. Each of these reagents has distinctly different properties because of the effect of the negative ion in the case of sulfuric acid, etc., and of the positive ion in sodium hydroxide, etc. The degrees of concentration of the active principle effected by the various combinations of these reagents are summarized in Table III.

Chemical Procedures

The material used in these studies was kindly furnished by Eli Lilly and Company and was prepared by them in the following manner. Rice polishings were thoroughly extracted with acidulated dilute ethyl alcohol. The alcohol was removed by concentration *in vacuo*, and the vitamin adsorbed on Lloyd's reagent. The activated earth was washed with water, and the vitamin eluted with sodium hydroxide. The alkaline solution was immediately acidified with sulfuric acid, and all the free sulfuric acid was neutralized by sodium hydroxide. A large part of the resulting sodium sulfate was precipitated by alcohol. The alcoholic vitamin solution was concentrated to a syrup and preserved with chloroform. In this condition it can be kept at room temperature for many months without apparent deterioration.

This vitamin concentrate was diluted until the amount of total solids (dried overnight at 100°) was about 30 per cent, and an aliquot part of this solution containing 2500 pigeon units (see Part I of this paper) was acidified with concentrated nitric acid to pH 4 (Congo red paper), and an excess of silver nitrate was introduced.[3] The resulting precipitate was washed by centrifuging, and the combined washings concentrated *in vacuo* (maximum tempera-

TABLE III

Concentration of Antineuritic Vitamin B with Silver under Various Conditions

Combination of reagents used			Biological assay			Degree of concentration calculated from		Vitamin recovered in active fraction
Silver	Acid	Alkali	Mean weight of birds	Total solids per pigeon unit*	N per pigeon unit*	Total solids	N	
			gm.	*mg.*	*mg.*			*per cent*
Original concentrate			331	27	1.3			
AgNO₃	HNO₃	Ba(OH)₂	295	7.2	0.4	4	3	10
"	"	NaOH	292	1.6	0.2	17	7	8
"	H₂SO₄	Ba(OH)₂	315	5.3	0.4	5	3	30
"	"	NaOH	299	6.2	0.5	4	3	5
"	Lactic acid	Ba(OH)₂	280	3.8	0.2	7	7	50
"	" "	NaOH	333	2.3	0.4	12	3	10
Ag lactate	H₂SO₄	Ba(OH)₂	285	6.4	0.3	4	4	50
" "	"	NaOH	352	2.6	0.3	11	4	10
" "	Lactic acid	Ba(OH)₂	298	6.0	0.3	5	4	30
" "	" "	NaOH	319	1.2	0.09	23	14	5

* Corrected to apply to a 300 gm. pigeon after the formula of Cowgill and Klotz (1927). The *pigeon unit* is the amount required to maintain the body weight constant over a period of from 10 to 14 days in the case of a 300 gm. pigeon fed according to the method described in this paper.

ture 30°) to the same volume as originally used. Hot saturated barium hydroxide was now added to about pH 7.0 as tested by brom-thymol blue. The precipitate was concentrifuged off and

[3] Excess silver was tested for (1) by the brown spot test with barium hydroxide, (2) by adding a drop of hydrochloric acid to the clear solution, and (3) by demonstration of silver in the filtrate after precipitation at pH 7.0.

washed[4] once by centrifuging with a little ice water. The precipitate was suspended in dilute hydrochloric acid (pH 1) and decomposed by boiling for ½ hour. The precipitation and washing of the vitamin never took more than 10 minutes. The silver chloride was removed and washed with warm water. The washings were concentrated to dryness *in vacuo*, and the residue was dissolved in about 100 cc. of water. Dilute sulfuric acid was added to precipitate all of the barium, and the barium sulfate centrifuged and washed. The washings were concentrated to dryness *in vacuo*, and the residue diluted to the same volume as had been originally employed. Aliquots of this solution were removed to determine total solids and nitrogen. The remainder was preserved with a trace of chloroform, and used for the feeding tests. The other experiments listed in Table III were carried out in essentially the same manner.

III. Solubility of the Antineuritic Vitamin in Certain Organic Solvents[5]

Since the report on the concentration of antineuritic vitamin B by fractional precipitation with ethyl alcohol by Osborne and Wakeman (1919), many attempts have been made to purify the vitamin by means of this solvent. There have been reports that the vitamin is not soluble in 99 to 100 per cent ethyl alcohol (Seidell, 1926; Seidell and Smith, 1930; Levene and van der Hoeven, 1925, 1926; Levene, 1928). Other investigators have reported solution of the active substance in absolute alcohol (Funk, 1911; Abderhalden and Schaumann, 1918; Kinnersley and Peters, 1927; Jansen and Donath, 1927; Guha and Drummond, 1929; Williams, Waterman, and Gurin, 1930). Van Veen (1931) attempted to concentrate the vitamin in the following ingenious fashion. He dried an active solution on powdered quartz and thoroughly extracted the finely ground sand with boiling absolute alcohol. However, this treatment failed to remove any appreciable amount

[4] A biological assay of this washing showed no vitamin present.
[5] Part of the work reported in Part III was done in the laboratories of the College of Physicians and Surgeons, Columbia University. One of us (R. J. B.) is indebted to Dr. Hans T. Clarke for the privilege of working in these laboratories and for assistance in securing special apparatus through a grant from the Chemical Foundation.

of the active substance.   These seemingly contradictory results
have been tentatively explained by Kinnersley and Peters (Sherman and Smith, 1931) thus: In the presence of some alcohol-insoluble impurities and at certain acidities, the vitamin is thrown out
upon alcoholic precipitation; after the removal of these substances,
it is soluble in alcohol of high concentration.   The writers believe
this suggestion to be incorrect because of the results of the experiments described below.

The object of these experiments was to prepare a solution of
antineuritic vitamin free from inorganic salts.   We therefore
attempted to remove these salts from a Lloyd's reagent concentrate prepared for us by Eli Lilly and Company according to the
procedures described above in Part II.   It was noticed that on
adding alcohol to the aqueous solution a large, flocculent gummy
precipitate appeared.   This precipitate could not be washed quantitatively and so resulted in a loss of from 50 to 60 per cent of the
active material.   However, it appeared to us that if the concentration of alcohol in the vitamin solution could be raised very
gradually from 50 to 100 per cent, there should be a complete
precipitation of the inorganic salts and other material insoluble
in absolute alcohol, the vitamin remaining in solution.   This
hypothesis was substantiated experimentally in the following
manner.

Solubility in Ethyl Alcohol-Carbon Tetrachloride

Through the kindness of Eli Lilly and Company, we obtained
three vitamin concentrates.   Two of these were prepared from
rice polishings and had been eluted from Lloyd's reagent with
sodium hydroxide and the alkali neutralized to pH 4 with sulfuric
acid.   The third (Concentrate III-A) was obtained from yeast
and had been eluted with sodium hydroxide and neutralized with
hydrochloric acid.[6]   The concentrates were diluted until the total
solid content was approximately 30 per cent.   Hydrochloric acid
was added to bring the reaction to pH 1 and any sulfuric acid
present was removed by addition of barium chloride.   The solution was heated on the steam bath and 5 volumes of a 1:1 solution
of 95 per cent ethyl alcohol and carbon tetrachloride were added;

[6] For a more detailed description of the preparation of these concentrates see Part II of this paper.

the water was then removed by conducting a distillation in the apparatus described in "Organic syntheses" (Adams, 1921). To compensate for the alcohol and carbon tetrachloride lost in the "water" layer, 10 cc. of carbon tetrachloride and 65 cc. of absolute alcohol were added for every 100 cc. of "water layer" distillate. The extraction was continued until no more water came over. The reaction flask was allowed to stand in the ice box overnight and the heavy precipitate filtered off. The filtrate was concentrated to dryness, a little water added and the solution again evaporated to dryness, this process being repeated several times in order

TABLE IV

Solubility of Antineuritic Vitamin in Binary Mixtures of Alcohol and Carbon Tetrachloride

Alcohol used	Total solids per pigeon per day	Degree of concentration of vitamin	Vitamin recovered
	mg.		*per cent*
Ethyl............................	48	3.5 ×	85
"	11	3.0 ×	100
"	7	4.8 ×	100
"	20	2.0 ×	85
n-Propyl.........................	40	1.0 ×	20
Isopropyl........................	26	1.5 ×	50
Allyl............................	25	1.7 ×	100
n-Butyl..........................	18	2.4 ×	100
Tertiary butyl....................	24	1.5 ×	25
" amyl...................	100	0	0

to remove excess hydrochloric acid. The final residue proved to be soluble in water, as well as in ethyl, *n*-propyl, isopropyl, allyl, and *n*-butyl alcohols. The vitamin is recovered practically quantitatively and the solution contains only the merest trace of inorganic material. The biological tests were made by one of us (G.R.C.) as a separate study according to the method described in Part I.

Solubility in Other Binary Mixtures

On account of the good results obtained with ethyl alcohol, it was considered advisable to attempt to concentrate the vitamin by employing other alcohols that form low boiling ternary mix-

tures with water and carbon tetrachloride. The following alcohols were tried: n-propyl, isopropyl, allyl, n-butyl, tertiary butyl, and tertiary amyl. The results, summarized in Table IV, indicate that only n-butyl and allyl alcohols are as good as or better than ethyl alcohol as a means of concentrating the antineuritic vitamin by this carbon tetrachloride process. Since the use of allyl alcohol is inadvisable on account of its high cost and its lacrimatory properties, ethyl and n-butyl alcohols remain as efficient solvents for concentrating the vitamin according to this technique.

Solubility in Certain Mixtures of Organic Solvents

The vitamin solution, which was purified according to the ethyl alcohol-carbon tetrachloride technique, was dissolved in absolute methyl alcohol. The alcoholic solution was then poured into 10 volumes of acetone, allyl, or amyl alcohol, and allowed to stand in the ice box for 24 hours. The distribution of the vitamin and the total solids in the filtrate and precipitate were then determined. The results indicate that no one of these mixtures can be used to advantage in concentrating the vitamin.

SUMMARY AND CONCLUSION

The method of assay for antineuritic vitamin B used in this investigation is described. It is a combination of weight-maintenance and curative techniques carried out on pigeons given a diet of polished rice ad libitum supplemented daily with meat residue, cod liver oil, and Osborne-Mendel salt mixture.

Ten experiments were carried out on the concentration of the antineuritic vitamin by means of silver. As sources of the silver ion, the nitrate and lactate salts were used; nitric, sulfuric, or lactic acid was employed as source of the hydrogen ion; and barium or sodium hydroxide was used for alkalizing. Silver nitrate, lactic acid, and barium hydroxide gave the most favorable purification with the least loss of activity; treatment with silver lactate, lactic acid, and sodium hydroxide resulted in the greatest increase of potency but in a poor yield of the vitamin.

The behavior of antineuritic vitamin in the ternary mixture of water, ethyl alcohol, and carbon tetrachloride was studied. Whereas the inorganic salts are precipitated as the water is re-

moved and the concentration of the alcohol approaches 100 per cent, it was found that the vitamin remains in the liquid phase from which it may be recovered quantitatively.

Solubility of the vitamin in other binary mixtures was studied. Six other alcohols were substituted for ethyl alcohol in the carbon tetrachloride procedure; of these only n-butyl and allyl proved to be as good as ethyl alcohol for concentrating the vitamin.

The solubility of the antineuritic vitamin in mixtures of methyl alcohol with acetone, allyl, and amyl alcohols was studied. No one of these mixtures can be used profitably to concentrate the vitamin.

BIBLIOGRAPHY

Abderhalden, E., and Schaumann, H., *Arch. ges. Physiol.*, **172**, 1 (1918).
Adams, R., Organic syntheses, New York, **1**, 68 (1921).
Burack, E., and Cowgill, G. R., *Proc. Soc. Exp. Biol. and Med.*, **28**, 750 (1930–31).
Carter, C. W., Kinnersley, H. W., and Peters, R. A., (a) *Biochem. J.*, **24**, 1832 (1930); (b) **24**, 1844 (1930).
Cowgill, G. R., *Am. J. Physiol.*, **79**, 341 (1926–27); **85**, 45 (1928).
Cowgill, G. R., and Klotz, B. H., *Am. J. Physiol.*, **81**, 470 (1927).
Cowgill, G. R., Rosenberg, H. A., and Rogoff, J., (a) *Am. J. Physiol.*, **96**, 372 (1931); (b) **98**, 589 (1931).
Eddy, W. H., Gurin, S., and Keresztesy, J., *J. Biol. Chem.*, **87**, 729 (1930).
Funk, C., *J. Physiol.*, **43**, 395 (1911–12); **45**, 75 (1912–13); *Proc. Soc. Exp. Biol. and Med.*, **25**, 133 (1927).
Funk, C., and Paton, J., *J. Metabol. Research*, **1**, 737 (1922).
Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, **41**, 297 (1926).
Guha, B. C., and Drummond, J. C., *Biochem. J.*, **23**, 880 (1929).
Hoagland, R., and Snider, G. G., *J. Agric. Research*, **40**, 977 (1930); **41**, 205 (1930).
Jansen, B. C. P., and Donath, W. F., *Proc. Konink. Akad. Wetensch. Amst.*, **29**, 1390 (1926).
Jansen, B. C. P., and Donath, W. F., *Mededeel. Dienst Volksgezonheid Nederland.-Indie*, **16**, 186 (1927).
Jansen, B. C. P., Kinnersley, H. W., Peters, R. A., and Reader, V., *Biochem. J.*, **24**, 1824 (1930).
Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, **19**, 820 (1925); **21**, 777 (1927); **22**, 419 (1928).
Klotz, B. H., Determination of the vitamin B minimum in the pigeon, Thesis, Yale University (1926).
Levene, P. A., *J. Biol. Chem.*, **79**, 465 (1928).

Levene, P. A., and van der Hoeven, B. J. C., *J. Biol. Chem.*, **65**, 483 (1925); *J. Pharmacol. and Exp. Therap.*, **29**, 227 (1926).

Myers, C. N., and Voegtlin, C., *J. Biol. Chem.*, **42**, 199 (1920).

Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **32**, 309 (1917).

Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, **40**, 383 (1919).

Peters, R. A., *J. State Med.*, **37**, 683 (1929); **38**, 3, 63 (1930).

Reader, V., *Biochem. J.*, **23**, 689 (1929); **24**, 77 (1930).

Seidell, A., *J. Biol. Chem.*, **29**, 145 (1917); *Pub. Health Rep., U.S.P.H.S.*, **37**, 1519 (1922); *J. Biol. Chem.*, **67**, 593 (1926).

Seidell, A., and Smith, M. I., *Pub. Health Rep., U.S.P.H.S.*, **45**, 3194 (1930).

Sherman, H. C., and Sandels, M. R., *J. Nutr.*, **3**, 395 (1930–31).

Sherman, H. C., and Smith, S. L., The vitamins, New York, 2nd edition (1931).

Smith, M. I., *Pub. Health Rep., U.S.P.H.S.*, **45**, 116 (1930).

Sullivan, M. X., and Voegtlin, C., *J. Biol. Chem.*, **24**, p. xvi (1916).

Van Veen, A. G., *Rec. trav. chim. Pays-Bas*, **1**, 208 (1931).

Vars, H. M., (1931) personal communication.

Williams, R. R., and Waterman, R. E., *J. Biol. Chem.*, **78**, 311 (1928).

Williams, R. R., Waterman, R. E., and Gurin, S., *J. Biol. Chem.*, **87**, 559 (1930).

IS BLOOD PROTEIN AMIDE NITROGEN A SOURCE OF URINARY AMMONIA?

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In a series of papers Bliss (1–5) has championed the view that neutralization of acid by ammonia is a generalized tissue process which contributes immediately to regulation of not only the acid-base equilibria of the organism as a whole but the local reaction in tissues of origin. Ammonia thus employed appears in the blood not as the salt of the acid but as an amide of blood protein from which, subsequently, the ammonia is liberated in the kidney. Consequently, urinary ammonia is ammonia which previously has been employed for neutralizing acids in remote tissues.

This theory of the mechanism and significance of ammonia metabolism is based essentially upon the following observations by Bliss: (1) an increase in protein amide nitrogen of the femoral venous blood following injection of ammonium carbonate into the companion artery; (2) the presence in kidney tissue of a deamidase[1] capable of liberating from blood proteins the same quantities of ammonia nitrogen as may be split off by acid hydrolysis under conditions calculated to affect only the amide groupings; (3) correlation of the level of blood protein amide nitrogen with acid production or administration and ammonia excretion in the urine.

We have undertaken herein to examine the experimental validity of these observations, and, for such as may be confirmed, to offer an alternative explanation.

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[1] No data have appeared describing the isolation, identification, and properties of this enzyme. Until such information is available any opinion upon its significance in ammonia metabolism is best reserved.

Measurements of Ammonia Nitrogen

Estimation of relatively large amounts of ammonia is one of the easiest, while precise measure of vanishing traces of ammonia is one of the most exacting, of analytical procedures. Since Bliss claims (2) for his results an experimental error not larger than 1 per cent, it is important to appreciate the absolute quantities of nitrogen involved in his analyses, and the degree to which experimental error may be magnified in the final result by the mode of expression employed.

The amounts of nitrogen taken for analysis are of the order of 0.3 mg. Measurement of this quantity within an error of 1 per cent permits an absolute error of not more than 0.003 mg. With the Bliss procedure the Nesslerized ammonia is contained finally in a volume of 50 cc., and thus the colorimetric comparison must detect a variation in color quantity of 1 part in 17 million. This borders very closely upon the limiting sensitiveness of the Nessler reaction. In our experience, the yellow shades of color are the most difficult to compare in the colorimeter. Even the slightest variations in quality of color, which occur unaccountably in duplicate Nesslerized mixtures, confuse the readings. When these color quality differences between standard and aerated unknown are intensified, as they frequently are by traces of caprylic alcohol or other less obvious factors, colorimetric comparison becomes only approximate. It is quite possible to read a standard color against itself, or to repeat several readings of the same unknown, within variations of less than 1 per cent. Visual memory is here an unavoidable factor. It is a much more uncertain matter to read identical colors with equal precision when it is not known in advance that the same solution is in both cups of the colorimeter. In our recent experience of several thousands of such readings we have not been confident of better individual accuracy than ±2 to 3 per cent. We are convinced that 1 per cent allowance does not suffice to cover consistently the subjective error[2] unavoidable in the final step alone of the Bliss method.

[2] In an effort to avoid the subjective errors of colorimetric measurements, we spent several months developing a micro titration technique which involved thiosulfate titration of iodine liberated from iodide-iodate mixture by the excess quantity of acid taken to receive the aerated ammonia. When ammonia may be distilled with steam and without the use of

Final results for amide nitrogen of the blood are expressed by Bliss as mg. per 100 gm. of protein. Since 0.2 cc. of blood is taken for analysis of protein amide nitrogen, a first factor of 500 is employed in the calculation. The resulting value is again multiplied, by the factor $\dfrac{100}{\text{gm. of protein per 100 cc. of blood}}$. Blood protein nitrogen is estimated in 0.01 cc. of blood, the nitrogen found being converted to gm. of protein per 100 cc. of blood by the factor 62,500. Obviously, small numerical differences among final values thus obtained must be interpreted with considerable reserve and strict attention to the absolute quantities represented by such differences in the original analyses. Thus, for example, should the entire experimental error of the analyses fall upon the protein amide nitrogen measurement, then, with an average blood and with the absolute quantities involved, a difference of 0.015 mg. of nitrogen (approximately 5 per cent) between duplicates would be magnified in the final expression to a difference of approximately 35 mg.

Methods and General Procedure

Except for certain modifications designed to improve the accuracy of the determination, we have followed the method for protein amide nitrogen essentially as described by Bliss (2).

Because of the rapid settling of corpuscles in dog blood, as well as the general difficulty of measuring accurate quantities of so viscous a fluid from delivery pipettes, we take 5 cc. of the well mixed blood in a pipette calibrated to contain. This is discharged into a 25 cc. volumetric flask and the pipette washed with water run in from the top. A small drop of ethyl alcohol may be used to dissipate the foam in the flask, the contents of which are then made to volume and thoroughly mixed. Delivery pipettes are used to take 5 cc. portions of this laked blood both for amide nitrogen and total nitrogen analyses.

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an antifoam agent, as in micro-Kjeldahl determinations, this titration is capable of the very highest precision. The necessary presence of a foam breaker in aerations, however, introduces difficulties which we have not yet entirely solved. For the work reported in the present paper, therefore, we have returned to colorimetric measurement.

For amide nitrogen determination the blood or plasma[3] proteins are precipitated in a 15 cc. centrifuge tube as directed by Bliss. Stirring rods with a small knob on the end are convenient, especially in the subsequent step of dissolving the protein in 0.9 N sodium hydroxide. In this step, both the stirring rod and the upper part of the centrifuge tube are scrubbed free of adherent protein by using the tip of the pipette containing the alkali, allowing a few drops of alkali to run out for this purpose before adding the remainder of it to the tube. The bulk of the precipitated protein dissolves rapidly in the 0.9 N alkali, but there is a small amount which dissolves finally only after some time and considerable rubbing with the end of the stirring rod. It should be noted that when the solution of alkali proteinate is finally made up to 10 cc. volume in the centrifuge tube, this can scarcely be done within a better accuracy than ±0.5 per cent, because of the relatively large diameter of the tube.

We have observed that if, during the first centrifugation after precipitating the blood proteins, the centrifuge is stopped after several minutes and the tubes are tapped and shaken gently, the slight film of protein which otherwise would remain in the surface is carried down by a second short period in the centrifuge. This precaution is not necessary when centrifuging the protein after the mother liquor has been decanted and the protein stirred up in dilute tungstic acid solution. It is thus possible to decant both mother liquor and tungstic acid washings into a 25 × 200 mm. Pyrex test-tube and determine the non-protein nitrogen by a micro-Kjeldahl digestion-distillation procedure. This manner of determining the non-protein nitrogen serves as a check upon the completeness of protein precipitation, and not only saves blood but would appear to be theoretically correct, since what non-protein nitrogen does not here appear will appear in part as protein amide nitrogen.

Of the 10 cc. solution of alkali proteinate, we have taken for hydrolysis 3 cc. portions, instead of 2 cc. portions as directed by Bliss. This reduces from 500 to 333 the factor by which the actual amount of nitrogen measured is converted to the basis of 100 cc. of blood. We have then added for the 90 minute hydro-

[3] 3 cc. portions of undiluted plasma are measured with pipettes calibrated to deliver, both for amide nitrogen and total nitrogen determinations.

lysis 1 cc. of 16 N sulfuric acid, in order to have the concentration of acid in the mixture 4 N as specified by Bliss. Before aeration, the hydrolyzed mixture is alkalinized by addition of 2 cc. of 10 N sodium hydroxide, thus giving in the aerated mixture the required alkalinity of $\frac{2}{3}$ N, but reducing the volume of the aerated mixture from 9 to 6 cc. In our experience, the smaller volume facilitates the removal of ammonia by the maximum aeration rate which may be employed without loss by spattering.

When sodium hydroxide is added to the strongly acid protein hydrolysate, even though the latter has first been cooled, the mixture becomes quite hot. If caprylic alcohol is present at this time, and aeration is begun while the temperature is still elevated, very large losses, up to 20 per cent, sometimes occur in the attempted transfer of known amounts of ammonia. We believe that under these conditions small amounts of aldehyde may be formed and combine with ammonia. Subsequent steam distillation of the aerated residue has invariably disclosed the missing ammonia. Other control tubes, aerated simultaneously in the same series, but cooled before receiving the alcohol, gave normal recoveries of ammonia. Our practice based upon this experience, therefore, is to connect the blood tube to the receiving, acid tube; place the blood tube in a small beaker of cool water; add the alkali to the blood tube and immediately close with its connection; mix the alkali by a few seconds aeration of the pair of tubes; then add 4 drops of caprylic alcohol through the longer, air inlet of the blood tube. The beakers of water are then removed, the various pairs of tubes connected in series, and aeration started.

Optimal conditions for transfer of ammonia by air current are probably an individual problem for each laboratory. Infinite attention to smallest details is essential to consistent results. After many weeks of tedious trial we determined upon the following rigid arrangement. One acid wash bottle and fifteen pairs of Pyrex 25 × 200 mm. tubes are aerated in series. The three sets of tubes distal from the water suction pump contain only acid and caprylic alcohol. They serve the threefold purpose of additional washing of the air; increasing the value of the negative pressure in the system; and reducing the rate of evaporation of caprylic alcohol from the intermediate tubes. If necessary, more alcohol may be added to these tubes through a stop-cock arrangement

during the course of aeration. The Pyrex tubes are slightly ground on the inner mouth to assure tight seating of the rubber stoppers which carry the air inlet and outlet connections. The air inlet connection of the acid-receiving tubes is blown out to a small bulb having a single small opening at the bottom approximately 0.5 mm. in diameter. With the resulting resistance to air flow through the system, aeration is carried out under appreciable partial vacuum. All air inlet connections extend as close as possible to the bottom of the tubes. U-shaped glass bends with short rubber connections join the alternate tubes in such manner that ammonia during transfer is in contact with a minimum amount of rubber surface. Air is drawn through the system by an efficient water pump, at moderate rate for the first 15 minutes, and then for 30 minutes at the maximal rate possible without loss by spattering.

With the above arrangement we aerate simultaneously triplicate samples of three bloods and three control quantities of standard ammonium sulfate solution, the twelve tubes being arranged haphazard in the series. Every value for protein amide nitrogen reported in our data has been obtained in this manner, with simultaneous control.

Following aeration and washing down of the connections of the acid-receiving tubes, caprylic alcohol is removed from the latter by 3 minutes boiling on a micro digestion shelf. Several glass beads are added to prevent bumping and mechanical loss in this step. Subsequently, the cooled contents of the tubes are transferred to 50 cc. volumetric flasks and a fixed quantity of Nessler's reagent added. Comparison is against a standard containing 0.45 mg. of nitrogen as ammonium sulfate.

Total nitrogen of blood and urine is determined in duplicate by the usual macro-Kjeldahl titration procedure. The 1 cc. quantities of blood employed are 100 times larger than the amount taken by Bliss for total nitrogen determination by his micro digestion-direct Nesslerization technique. In all cases we have corrected the total nitrogen of blood for the non-protein nitrogen, in reporting total protein nitrogen.

Except in a few instances, urinary ammonia has been measured by aeration of duplicate 25 cc. portions of the appropriately diluted urine, with back titration of the 0.1 N receiving acid. With several

very small urine volumes ammonia was determined by aeration of smaller duplicate quantities, and Nesslerization.

All urines were taken by catheter, with thorough and repeated washing of the bladder with water. The water washings were combined with the respective urines. Urine periods as given are accurate within 30 seconds.

Pipettes or other volumetric apparatus employed were carefully checked in the laboratory.

Total nitrogen analyses of blood and urine, and urine ammonia determinations, were made by the junior author. Protein amide nitrogen, non-protein nitrogen, and ammonia were determined for the blood by the senior author. The two sets of results, which in some cases are merged into a single final value, were thus accomplished entirely independently.

In Table I are assembled the values of analyses in triplicate of protein amide nitrogen of the forty-four bloods which are cited in Tables II, IV, VII, and IX. These data represent the best average accuracy and consistency we have been able to accomplish after long practice with the method employed. It is noteworthy that, among triplicates, occasionally two may differ by as much as 8 per cent. Average agreement is much better than this. Inspection of the forty-four groups of values discloses that the maximum per cent deviation from mean value within any group is 4.5 per cent, and the average of all deviations is 1.17 per cent. We believe that no significance can be attached to any individual value of blood protein amide within a variation of less than 5 per cent. The general trend of amide values, however, in one or a series of experiments may be interpreted less conservatively.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Ammonia Injections and Protein Amide Synthesis*</div>

Demonstration beyond doubt that tissues do synthesize protein amides from injected ammonia is, according to Bliss (3), a point of "cardinal importance" to his thesis. Before inspecting his data which are interpreted as demonstrating this process, it may be noted that the simple fact of an animal tolerating *via* femoral artery a larger quantity of ammonium carbonate than can be given by vein without inducing symptoms of ammonia poisoning is of

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Protein amide N	Deviation from mean value	Protein amide N	Deviation from mean value	Protein amide N	Deviation from mean value	Protein amide N	Deviation from mean value	Protein amide N	Deviation from mean value	Protein amide N	Deviation from mean value
Dog 4		Dog 6		Dog 8		Dog 10		Dog 13		Human, mixed	
mg. per 100 cc.	per cent	mg. per 100 cc.	per cent	mg. per 100 cc.	per cent	mg. per 100 cc.	per cent	mg. per 100 cc.	per cent	mg. per 100 cc.	per cent
138.4	0.43	127.1	0.71	146.6	1.74	137.8	0.44	131.8	0.23	122.5	0.41
141.7	1.94	127.3	0.87	151.6	1.61	136.6	0.44	131.2	0.23	122.0	0.00
136.8	1.58	124.1	1.67	149.3	0.07	134.9	0.30	131.6	0.08	121.4	0.49
148.5	3.34	124.4	0.40	145.0	2.76	133.3	1.48	120.6	1.69		
140.6	2.16	121.6	2.64	141.0	0.07	137.8	1.85	117.8	0.67		
141.9	1.25	128.6	2.96	137.4	2.62	123.1	1.65	117.5	0.93		
129.4	2.45	124.3	3.50	138.9	1.56	119.6	1.24	119.5	1.97		
124.5	1.42	120.3	0.17	142.3	0.85	120.7	0.33	121.8	0.08		
124.9	1.11	115.6	3.75	142.1	0.71	129.3	0.86	124.5	2.13		
128.8	2.35	121.1	0.83	135.9	0.74	128.0	0.16	127.7	1.75		
132.8	0.68	119.6	0.42	134.8	0.07	127.3	0.70	127.1	1.28		
134.0	1.59	119.7	0.33	133.9	0.74	128.9	0.46	121.8	2.95		
Dog 5		115.5	0.96	142.7	0.14	132.0	1.93	128.0	0.95		
123.4	3.61	113.7	0.61	143.7	0.56	127.5	1.54	124.8	1.58		
116.6	2.10	114.1	0.26	142.4	0.35	128.7	0.00	127.7	0.71		
117.4	1.43	115.9	0.00	141.8	0.77	128.0	0.54	123.8	1.14		
106.8	1.02	116.9	0.86	143.7	0.56	129.5	0.62	124.7	1.88		
112.8	4.54	115.0	0.78	142.9	0.00	122.4	1.05	118.7	3.02		
104.2	3.43	Dog 7		Dog 9		123.8	0.08	123.0	1.15		
103.2	2.08	126.4	0.79	130.6	0.62	124.8	0.89	121.3	0.25		
106.0	0.57	128.0	0.47	129.2	0.46	122.3	1.21	120.5	0.90		
107.0	1.52	127.7	0.24	129.7	0.08	126.2	1.94	124.1	1.06		
105.2	0.67	135.6	0.07			122.8	0.81	122.3	0.41		
103.9	0.57	137.0	0.96					122.0	0.65		
		134.6	0.81					120.8	2.46		
		151.5	2.30					116.7	1.02		
		143.8	2.91					116.1	1.53		
		149.1	0.68					120.3	1.78		
		137.4	0.29					118.1	0.08		
		135.9	1.38					116.3	1.61		
		140.1	1.67								
		142.1	0.07								
		145.4	2.25								
		139.2	2.11								

itself not the slightest evidence that the muscle tissue has disposed of any part of the ammonia by synthesis. We should expect that a substance thus injected would be held up in the capillary bed of the muscle and reach the general circulation over a longer period and thus in smaller concentration than if it had been given by vein. This is true, for example, with adrenalin administration. The margin of tolerance found by Bliss as between artery and vein is indeed smaller than we should have anticipated. He reports that 20 mg. of ammonia nitrogen per kilo of body weight will induce symptoms when put into the vein, whereas up to 24 mg. per kilo

TABLE II

Effect of Ammonium Carbonate Injection upon Blood Protein Amide Nitrogen

Dog 7, female; weight, 20.1 kilos. Fasted 40 hours. 12 per cent ammonium carbonate (29.3 mg. of N per cc.) was injected into the femoral artery (19 mg. of N per kilo). Sodium amytal anesthesia was given, with subsequent good recovery.

Time after ammonium carbonate injection	Femoral vein					Femoral artery			
	NH_3-N per 100 cc.	Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N		Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N	
				Per 100 cc.	Per 100 gm. protein			Per 100 cc.	Per 100 gm. protein
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Preliminary period		28.9	2894	127.4	704	32.7	3097	135.7	701
13.0 cc. in 4½ min.									
1¾ min.	19.9	53.4	3254	148.1	728	33.2	3207	137.8	688
10 "	3.2	35.6	3166	142.2	719				

can be given *slowly via* femoral artery without symptomatic reactions. It appears from these figures alone that, at most, only a small fraction of the amount injected by artery could be employed in amide synthesis. In the one experiment of this sort which we have performed (Table II) we found, as expected, large amounts of ammonia in the femoral vein not only during and immediately following injection into the artery, but for some time afterward. That is to say, ammonia remains in the muscle tissue and should be available for amide synthesis far beyond the few seconds during which Bliss observed even debatable percentage increases in protein amide nitrogen of the venous blood.

The data offered by Bliss in proof of amide synthesis of injected ammonia are contained in nine experiments (3, 5), four of which serve as controls.   12 per cent ammonium carbonate solution in amount representing 20 mg. of nitrogen per kilo was injected into the femoral artery of five dogs, the time required for injection varying from $1\frac{1}{2}$ to $4\frac{1}{2}$ minutes.   Corresponding injections of 12 per cent sodium carbonate, adjusted with CO_2 to the same pH as the ammonium carbonate, were given to the four controls.   Samples of blood were taken from artery and vein simultaneously, before and immediately following injection, and three or four additional collections were made at approximately 2 minute intervals.   Comparison of the protein amide nitrogen values of

TABLE III

Data Taken from Bliss' Experiments (3, 5) upon Blood Protein Amide Synthesis from Injected Ammonium Carbonate

The values represent venous minus arterial differences, and are expressed as mg. of N per 100 cc. of blood.

Ammonium carbonate injected					Controls, sodium carbonate injected			
Dog 31	Dog 32	Dog 37	Dog 38	Dog 56	Dog 32	Dog 34	Dog 35	Dog 37
+6	+8	+14*	+14*	+6	0	+6	+4	−12*
+2	+5	+7	+3	+3	−2	+5	+4	0
+3	+3	+3	−2		+4	+3	+4	+2
+2	+3	+3	−1	−3	+2	+2	+3	+5
−2	+6	+4	−6	0		+3	+5	+3

arterial and venous bloods following injection has been condensed into Table III, the values being expressed as mg. of nitrogen per 100 cc. of blood, and representing venous minus arterial differences.

Of the forty-three values in Table III we believe that only the three we have marked with an asterisk (each of which occurs within 20 to 90 seconds after injection) are larger than the probable experimental error.   It is interesting that if these three values be omitted the algebraic sums of the remaining values in each of the two groups of dogs are identical.   We agree with Mann (6) that such data do not demonstrate synthesis of protein amide from injected ammonia.

It is, perhaps, unnecessary to account for three divergent results in a series of forty-three.   In view of the grossly unphysiologic

character of 12 per cent ammonium or sodium carbonate we should be disposed to regard blood or other tissue changes subsequent to injection of such materials as pathologic manifestations rather than intensified normal processes. Bliss, indeed, finds ammonium carbonate so irritating that he is careful to remove any ammonia adhering to the outside of the needle used for the intraarterial injection. Despite this precaution, we have seen very definite local reaction by the artery during injection. Profound and unpredictable effects upon endothelium and capillary permeability may be produced. Changes in the protein concentration of the blood, as well as in the distribution among the several protein fractions, might well be anticipated. We have observed (Table IV) that the amide nitrogen value of the plasma protein is from 30 to 35 per cent larger than for the whole blood protein. This fact is in accord with the much larger proportion of glutamic and aspartic acids constituting serum albumin and serum globulin as compared with hemoglobin (7). It would seem, therefore, that water (and, conceivably, protein) exchange between plasma and lymph affords a ready explanation not only of changes in blood protein concentration but of variations in percentage of whole blood protein amide, if such changes are, in fact, demonstrable.

In only one of his experiments upon ammonium carbonate-injected dogs did Bliss follow the protein values (5). No considerable change was found except in the arterial blood drawn 20 seconds after the injection. Here the protein nitrogen was 3.45 as against 3.23 per cent before injection. Less than 2 minutes later the value had fallen to 3.33 per cent. Such fleeting and random changes in the *arterial* blood are entirely unaccountable in any part of Bliss' theory. In frequent other instances it is a change in absolute arterial values which is mainly responsible for calculated relative increases in venous values. For example, the "−12" value for Dog 37 (Table III) results 1 minute after injection from an increase of protein amide nitrogen in the arterial blood from 131 to 138 mg. per cent, and the simultaneous decrease in vein from 134 to 126 mg. per cent. It would seem fully as important to account for absolute changes in arterial values as to emphasize relative venous changes, since, of course, arterial blood, and not venous blood, mirrors the immediate conditions with which the kidney has to deal.

In our experiment upon Dog 7 (Table II) significant increases in protein concentration of the blood of both femoral vein and artery appear immediately following injection of ammonium carbonate into the artery. The increase above preliminary value is in vein 12.5 per cent and in artery 3.5 per cent. Concomitantly, the amide nitrogen per 100 gm. of protein decreases 2 per cent in the artery and increases 3 per cent in the vein. Thus, the preliminary "venous minus arterial" difference of +3 mg. becomes +40 mg. per 100 gm. of protein. This is the character of change interpreted by Bliss as synthesis of protein amide from the injected ammonia, but taken by us as well within the range of experimental error or, if valid, as explicable in terms of fluctuation in relative quantity

TABLE IV

Protein Amide Nitrogen of Whole Blood and Plasma

Potassium oxalate served as anticoagulant.

Sample	Whole blood				Plasma			
	Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N		Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N	
			Per 100 cc.	Per 100 gm. protein			Per 100 cc.	Per 100 gm. protein
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Jugular vein, Dog 8........	33.7	3306	142.9	692	18.6	1000	57.7	923
Median cubital vein, human, mixed....................	25.7	2920	122.0	669	20.6	1193	67.7	908
Femoral artery, Dog 9......	27.4	2816	129.8	737	16.6	1105	67.4	976

of the individual blood proteins. We have cited the large difference in amide nitrogen content as between plasma protein and hemoglobin. Considerable variations in the relative amounts of the several protein fractions of blood occur normally as well as under pathological conditions, and inevitably must affect the amide nitrogen value as determined for the whole blood. We conceive that ammonium carbonate injection may bring about such a change in distribution among the several protein fractions of the venous blood as to account for the results obtained in Dog 7. The prompt, though smaller, increase in protein concentration of the arterial blood is, likewise, attributable to effects produced throughout the organism by ammonia which, at this time, had

been circulating in the systemic blood for 6 minutes. Since, in this experiment, our original interest was in demonstrating the persistence of ammonia in the muscle and its vein after injection into the artery, we were not prepared to follow the arterial blood beyond the immediate after period. It is our purpose, however, to study further and in greater detail the blood changes following ammonium carbonate administration in an effort to determine whether they are consistent and demonstrably due to changes in quantity and not quality of the individual proteins.

Effect of Acid Administration upon Blood Protein Amide

Bliss concedes that a crucial test of the acid-neutralizing rôle of ammonia in general tissue metabolism consists in demonstrating a parallelism between increases in urinary ammonia and increases in blood protein amide nitrogen. He says (5), "A demonstrable increase of acid in the blood, and which causes an increase of ammonia in the urine, must result in an increase in the *blood amides*." His evidence upon this relationship is supplied by observations upon dogs which have exercised for 2 to 10 minutes, or have received by stomach tube such quantities of hydrochloric acid as to increase markedly the urinary ammonia.[4] In Table V we have summarized from the data of these experiments the protein amide nitrogen values of the arterial blood.

The most casual inspection of Table V reveals that there is no consistent increase in the level of protein amide nitrogen of arterial blood during periods when, presumptively or demonstrably, the dogs were excreting increased amounts of ammonia in the urine. The trend, if there is one in these figures, is more in the direction of a decreased concentration of arterial protein amide. Of the 54 values, nineteen are higher, and thirty-four are lower than the preliminary values, while one value is unchanged. Furthermore the variations occur at random, and show no consistent relationship with time intervals in the experiments.

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[4] In his several experiments upon phlorhizinized dogs Bliss has withheld the data upon actual concentration of amide nitrogen in the blood, and is content to base his conclusions upon the value of amide nitrogen per 100 gm. of protein. We would emphatically restate our conviction as expressed in the main body of our paper that the proper value for discussion is absolute concentration per unit volume of blood; the kidney is supplied with fluid blood and not with 100 gm. batches of solid protein.

Dog 8 (Table V) had received large amounts of hydrochloric acid during the 4 days preliminary to the exercise experiment. Bliss interprets the blood values subsequent to exercise of this dog as confirming that the preliminary acid administration had so diminished a fixed supply of ammonia or its precursor in muscle that the lactic acid formed in exercise was neutralized almost wholly by fixed base and the amount of amide nitrogen given up by muscle

TABLE V

Data Taken from Bliss' Experiments (3, 5) upon Effect of Acid Administration or Exercise upon Blood Protein Amide Nitrogen

All values are for arterial blood, and represent mg. of protein amide N per 100 cc. of blood.

	Exercise									Acid administration		
	Dog 8*	Dog 4†	Dog 28†	Dog 5†	Dog 9†	Dog 21‡	Dog 22§	Dog 12‖	Dog †	Dog 81¶	Dog 69**	Dog 80**
Preliminary period	160	162	155	155	155	154	141	150	148	156	136	131
Post exercise or post acid period	159	163	147	148	152	155	146	157	140	150	137	136
	157	163	146	153	148	155	142	149	150	152	134	135
	150	158	150	144	144	149	137	157	149		133	136
	158	156	155	152	145		126	156	147		134	
	157	163	162	149	147	134	125	158	153			
							129					

* 2 minutes exercise after 4 days of acid administration.

† 2 minutes exercise.

‡ 5 minutes exercise.

§ 10 minutes exercise.

‖ 2 minutes exercise. Dog with fixed alkali plethora.

¶ 300 cc. N/7 HCl.

** 350 cc. N/7 HCl.

was correspondingly less than normal. This view does not appear to be in harmony with the fact that the absolute concentration of protein amide nitrogen in the blood of this dog was at the upper limit observed in the entire series.

The experiment upon Dog 9 was the only one in the exercise group for which ammonia excretion in the urine was determined. During the first 20 minutes after exercise the NH_3-N excretion in the urine was at the rate of 23.4 mg. per hour, while the arterial

protein amide nitrogen values during this period were, consecutively, 152, 148, and 144 mg. per cent. For the subsequent 40 minute period the excretion rate was 14.1 mg. of NH_3-N per hour, with the blood values in two samples at 145 and 147 mg. per cent. In other words, the maximum rate of ammonia excretion coincides with the lowest concentration in the blood of the substance which Bliss represents as transporting to the kidney in "detoxified form" the increased quantities of ammonia produced in muscle. Precisely the same paradox appears in at least two of the three experiments in which acid was given and ammonia excretion was followed. In both Dogs 69 and 81 increases in rate of ammonia

TABLE VI

Data Taken from Bliss' Experiment upon Dog 69 (5), Illustrating Mode of Expressing Final Results Which Translates Absolute Decreases in Blood Protein Amide Nitrogen into Percentage Increases

	Arterial blood		
	Total N	Protein amide N	
		Observed per 100 cc.	Calculated per 100 gm. protein
	per cent	*mg.*	*mg.*
Preliminary period	2.83	136	767
After acid administration	2.81	137	779
	2.72	134	790
	2.68	133	796
	2.53	134	848

excretion of the order of 3 to 5 times the initial rate occur during periods when the concentration of protein amide in the arterial blood is, if changed at all, below the preliminary value.

However, the mode of interpretation of his data which Bliss has adopted in his latest paper (5) enables absolute decreases in blood protein amide to be transformed into percentage increases. This method is illustrated in the tabulation of data from his experiment upon Dog 69, which we have condensed in Table VI. Taking these data as typical, Bliss says:

"Dog 69 . . . was given 350 cc. of seventh normal hydrochloric acid by stomach tube. The blood amide nitrogen, starting from a preliminary value of 767 mgm., rose to 779, 790, 796 and 848 mgm. at hourly intervals

after acid administration. These progressive rises in the values for blood amides occurred in the middle of hourly periods of urine collections that showed corresponding progressive rises in urinary ammonia elimination. The values for urinary ammonia rose from a preliminary value of 13.6 mgm. per hour to 19.8, 25.5 and 30 mgm. respectively. At the time when the blood amides were rising along with the increase in the excretion of urinary ammonia, the ratios of total nitrogen to ammonia-nitrogen were falling, and this is additional evidence *that these rises in the values for blood amides occurred at a time when extra ammonia was being used for acid neutralization."*

This method of manipulating experimental data, and such statements based upon its use, seem to us to be altogether misleading and unjustified. It is apparent that the "increases" cited are due to unexplained *decreases* in concentration of blood protein, and that the "increases" would be 10 times as large, if not ten times as convincing, were the results calculated to the basis of 1000 gm. of protein.

If the rate of ammonia excretion by the kidney in any degree varies with the concentration of protein amide in the blood, it is essential to invoke some new concept of renal threshold behavior to reconcile an increased rate of ammonia elimination with an absolute decrease in concentration of blood protein amide. Were the percentage values found by Bliss in this instance typical, one might perhaps assume that the relatively increased amide groupings were in more labile state than is true for the normal protein molecule, and that the renal threshold for ammonia is measured in terms of the concentration of this *labile* amide nitrogen. This notion, however, is no part of Bliss' theory; on the contrary, he has at least in one place (1) definitely inferred that all of the amide nitrogen of the blood proteins has the same significance. We have found, in fact, that increases in blood protein amide nitrogen, either absolute or relative to the blood protein, *do not* ordinarily occur in periods subsequent to acid administration and coincident with largely increased rate of ammonia excretion in the urine.

Data of our experiments upon the relationship between rate of ammonia excretion and level of blood protein amides are given in Table VII. In two of the five dogs employed (Dogs 10 and 13) the acid experiment was controlled by water administration on the preceding or following day, other conditions being identical. Dogs 4, 5, and 8 received a relatively low protein diet of dog biscuit which produced normally a slightly acid or neutral urine. Dogs 10

Effect of Acid or Water Administration upon Blood Protein Amide Nitrogen and Ammonia Excretion

Time	Blood, femoral artery				Urine					
	Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N		Volume	Total N		NH₃-N		Ratio total N / NH₃-N
			Per 100 cc.	Per 100 gm. protein		For period	Per hr.	For period	Per hr.	

Dog 4, female; weight 13.1 kilos. Diet, Champion dog biscuit. No anesthesia

Time	Non-protein N	Protein N	Per 100 cc.	Per 100 gm. protein	Volume	Total N for period	Per hr.	NH₃-N for period	Per hr.	Ratio
1931	*mg.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	
May 23										
9.18–11.18 a.m.					22	245	123	9.5	4.8	26
10.26 a.m.	24.5	3055	139.0	728						
300 cc. N/7 HCl at 11.20 a.m.										
11.18 a.m.–1.18 p.m.					148	499	250	49.3	24.6	10
12.18 p.m.	24.2	3031	143.7	759						
1.18–3.18 p.m.					152	459	229	64.1	32.0	7
2.18 p.m.	22.9	2930	126.3	690						
3.18–5.18 p.m.					43	351	175	48.5	24.3	7
4.18 p.m.	21.9	2884	131.9	732						

Dog 5, female; weight, 12.5 kilos. Diet, Champion dog biscuit. No anesthesia

Time	Non-protein N	Protein N	Per 100 cc.	Per 100 gm. protein	Volume	Total N for period	Per hr.	NH₃-N for period	Per hr.	Ratio
May 28										
10.08 a.m.–12.08 p.m.					13	565	283	6.1	3.0	93
10.45 "	32.7	2525	119.1	754						
300 cc. N/7 HCl at 12.11 p.m.										
12.08–2.08 p.m.					121	908	454	49.9	25.0	18
1.08 p.m.	33.1	2397	107.9	720						
2.08–4.08 p.m.					44	876	438	71.2	35.6	12
3.08 p.m.	30.9	2374	105.4	710						
4.08–6.08 p.m.					25	730	365	66.0	33.0	11
5.08 p.m.	28.3	2416	104.5	692						

Dog 8, female; weight, 12.3 kilos. Diet, Champion dog biscuit. No anesthesia

Time	Non-protein N	Protein N	Per 100 cc.	Per 100 gm. protein	Volume	Total N for period	Per hr.	NH₃-N for period	Per hr.	Ratio
June 23										
11.30 a.m.–1.30 p.m.					10	266	133	2.2	1.1	121
1.15 p.m.	34.2	3348	149.2	713						
300 cc. N/7 HCl at 1.33 p.m.										
1.30–3.30 p.m.					18	394	197	14.3	7.2	28
2.30 p.m.	31.9	3170	141.1	712						
3.30–5.30 p.m.					36	389	195	30.9	15.5	13
4.30 p.m.	28.1	3172	141.1	712						
5.30–7.30 p.m.					56	384	192	36.2	18.1	11
6.30 p.m.	30.3	3002	134.9	719						

TABLE VII—*Continued*

Time	Blood, femoral artery				Urine					
	Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N		Volume	Total N		NH₃-N		Ratio total N / NH₃-N
			Per 100 cc.	Per 100 gm. protein		For period	Per hr.	For period	Per hr.	

Dog 10, female; weight, 12.8 kilos.   Diet, 400 gm. cooked beef heart daily. No anesthesia

1931	mg.	mg.	mg.	mg.	cc.	mg.	mg.	mg.	mg.	
July 16										
10.45 a.m.–12.45 p.m.					18	708	354	36.1	18.1	20
11.47 "	37.4	3022	137.2	726						
350 cc. N/7 HCl at 12.48 p.m.*										
12.45–2.45 p.m.					55	829	414	60.6	30.3	14
1.46 p.m.	35.2	3011	135.3	719						
2.45–4.45 p.m.					100	724	362	76.0	38.0	10
3.45 p.m.	31.1	2734	121.1	709						
4.45–6.45 p.m.					36	577	288	60.6	30.3	10
5.45 p.m.	29.5	2890	128.2	710						
July 17										
10.50 a.m.–12.50 p.m.					25	870	435	68.3	34.1	13
12.00 m.	37.4	2904	129.5	714						
300 cc. water at 12.53 p.m.										
12.50–2.50 p.m.					212	858	429	62.2	31.1	14
1.50 p.m.	32.8	2939	128.7	701						
2.50–4.50 p.m.					42	634	317	49.0	24.5	13
3.50 p.m.	31.2	2758	123.7	718						
4.50–6.50 p.m.					20	513	257	41.3	20.7	12
5.50 p.m.	28.2	2766	123.8	716						

Dog 13, female; weight, 15.3 kilos.   Diet, 500 gm. cooked beef heart daily. No anesthesia

July 20										
9.40–11.40 a.m.					27	1209	605	79.4	39.7	15
10.45 a.m.	50.5	2817	131.5	747						
400 cc. water at 11.43 a.m.										
11.40 a.m.–1.40 p.m.					200	1390	695	78.0	39.0	18
12.42 p.m.	42.6	2613	118.6	726						
1.40–3.40 p.m.					55	956	478	71.5	35.8	13
1.44 p.m.	40.2	2722	121.9	717						
2.40 "	38.5	2634	125.5	762						
3.40–5.40 p.m.					45	965	482	66.3	33.1	15
4.40 p.m.	36.2	2634	126.8	770						

TABLE VII—*Concluded*

Time	Blood, femoral artery				Urine					
	Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N		Volume	Total N		NH₃-N		Ratio total N / NH₃-N
			Per 100 cc.	Per 100 gm. protein		For period	Per hr.	For period	Per hr.	

Dog 13, female; weight, 15.3 kilos.   Diet, 500 gm. cooked beef heart daily. No anesthesia—*Concluded*

1931	mg.	mg.	mg.	mg.	cc.	mg.	mg.	mg.	mg.	
July 21										
9.30–11.30 a.m.					78	1499	749	97.8	48.9	15
10.35 a.m.	52.0	2685	122.4	730						
400 cc. N/7 HCl at 11.34 a.m.†										
11.30 a.m.–1.30 p.m.					157	1557	779	115.8	57.9	13
12.30 p.m.	45.6	2604	121.6	747						
1.30–3.30 p.m.					204	1418	709	134.4	67.2	11
1.33 p.m.	45.1	2587	122.8	759						
2.30 "	40.8	2525	117.9	747						
3.30–5.30 p.m.					180	1223	611	133.0	66.5	9
4.30 p.m.	35.3	2524	118.2	749						

* Dog 10 vomited within the first half-hour after receiving acid.   Vomitus titrated equivalent to 62 cc. of N/7 HCl.

† Dog 13 drank freely of water in the cage after receiving acid.

and 13 were fed a high protein diet of cooked beef heart which produced strongly acid urine.   Feedings were once daily, and in all cases between 5.00 and 7.00 p.m. on the day preceding experiment.   Acid or water was given by stomach tube in one dose. Water was available in the cages throughout.

What we take to be a dilution effect of absorbed fluid is clearly evident both in the non-protein and protein nitrogen values of the blood, which fall progressively from control levels.   Within the limits of experimental accuracy, the protein amide values parallel changes in blood protein concentration.   Even when expressed as mg. per 100 gm. of protein, the trend of the amide nitrogen values is rather slightly downward than upward.   Certainly, in these experiments, we find no confirmation of the "increases" in blood amide nitrogen reported by Bliss to follow acid administration.

Particularly instructive are the data upon Dogs 10 and 13.   For each of these dogs the blood data of the day of acid administration duplicate with surprising exactness the blood findings for the control day of water administration.   In each case, by contrast, the curve of rate of ammonia elimination following acid administration is an inversion of that following water.   Increases up to 100 per cent in rate of ammonia excretion, or decreases of nearly comparable magnitude, occur indifferently with stationary or lowered levels of blood protein amides.

Effect of Exercise upon Ammonia Excretion

Bliss has relied upon a single observation, uncontrolled by fore or after periods, and an unwarranted inference from the work of Liljestrand and Wilson (8), for his conclusion that brief periods of moderate exercise in the dog induce an immediate increase in rate of ammonia elimination in the urine.   His experiment upon Dog 9, and the urine ammonia values during the hour following 2 minutes of exercise by this dog, have been cited in the preceding section.   Of these findings Bliss says (3): "Since these results were in agreement with the very complete and convincing work of Liljestrand and Wilson . . . , no other urinary analyses were attempted."   The work of Liljestrand and Wilson cited was upon the excretion of lactic acid in the urine after exercise in the *human*, and we have not found in a careful reading of their paper any data whatsoever upon ammonia.

Various considerations argue against the probability that short exercise by the dog could induce so immediate and considerable increases in urinary ammonia as Bliss has assumed.   Rice and Steinhaus (9), for example, have shown recently that, in the exercised dog, there is a temporary increase in the pH of the blood. The mechanism of this effect is explained by Rice and Steinhaus as overventilation, since the dog, with practical absence of sweat glands, is obliged to eliminate much of the extra heat arising from exercise by evaporation of water from lungs and tongue.

If one immediate effect of exercise is a temporary increase in alkalinity of the blood, it might be predicted from the work and theory of Nash and Benedict (10) that a secondary effect of exercise would be a *decreased* rate of ammonia excretion in the urine.

To test this hypothesis we have carried out the experiments which are summarized in Table VIII.

TABLE VIII

Effect of Exercise upon Ammonia Excretion in Urine

Time	Urine							Ratio $\frac{\text{total N}}{\text{NH}_3\text{-N}}$
	Volume	Reaction to litmus	Total N		NH$_3$-N			
			For period	Per hr.	For period	Per hr.		

Dog 10, female; weight, 12.8 kilos. Diet, Champion dog biscuit. Last feeding, 10.00 a.m., July 1. By stomach tube, 250 cc. water at 12.18 p.m., and 100 cc. water at 2.18 p.m., July 2

1931	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*		
July 2, p.m.								
12.15–1.15	28	Neutral	161	161	3.3	3.3	49	
1.15–2.15	140	"	190	190	8.1	8.1	23	
Exercise, 2.19–2.23								
2.15–2.45	6	"	51	101	0.9	1.7	59	
2.45–3.15	21	Basic	117	234	1.2	2.5	95	
3.15–4.15	87	Neutral	192	192	7.6	7.6	25	

Dog 9, female; weight, 16.8 kilos. Diet, Champion dog biscuit. Last feeding, 6.30 p.m., July 5. By stomach tube, 300 cc. water at 1.14 p.m., and 100 cc. water at 3.13 p.m., July 6

July 6, p.m.								
1.11–2.11	48	Acid	119	119	6.5	6.5	18	
2.11–3.11	71	Neutral	91	91	5.4	5.4	17	
Exercise, 3.14–3.18								
3.11–3.41	7	Basic	30	60	1.7	3.4	18	
3.41–4.11	29	Neutral	59	118	2.8	5.6	21	
4.11–5.11	60	"	85	85	4.6	4.6	19	

Dog 11, female; weight, 10.9 kilos. Diet, 350 gm. cooked beef heart daily. Last feeding, 6.00 p.m., July 8. By stomach tube, 250 cc. water at 12.46 p.m., and 100 cc. water at 1.48 p.m., July 9

July 9, p.m.								
12.43–1.43	26	Acid	318	318	23.1	23.1	14	
1.43–2.43	134	"	311	311	23.2	23.2	13	
Exercise, 2.44–2.48								
2.43–3.13	2	"	39	77	4.7	9.4	8	
3.13–3.43	4	"	153	306	6.4	12.8	24	
3.43–4.43	34	"	259	259	14.7	14.7	18	

TABLE VIII—*Concluded*

Time	Volume	Reaction to litmus	Total N		NH₃-N		Ratio $\frac{\text{total N}}{\text{NH}_3\text{-N}}$
			For period	Per hr.	For period	Per hr.	

Dog 10, female; weight, 12.9 kilos. Diet, 400 gm. cooked beef heart daily. Last feeding, 5.30 p.m., July 9. By stomach tube, 300 cc. water at 2.48 p.m., and 100 cc. water at 4.47 p.m., July 10

Time	Volume	Reaction to litmus	Total N For period	Total N Per hr.	NH₃-N For period	NH₃-N Per hr.	Ratio
1931	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*	
July 10, p.m.							
2.45–3.45	92	Acid	390	390	21.1	21.1	19
3.45–4.45	81	"	366	366	21.1	21.1	17
Exercise, 4.51–4.54							
4.45–5.15	19	Neutral	176	352	8.1	16.2	22
5.15–5.45	19	"	166	332	8.2	16.3	20
5.45–6.45	23	Acid	308	308	15.8	15.8	20

Dog 12, female; weight, 19.7 kilos. Diet, 400 gm. cooked beef heart daily. Last feeding, 7.00 p.m., July 10. By stomach tube, 400 cc. water at 12.44 p.m., and 150 cc. water at 2.38 p.m., July 11

Time	Volume	Reaction to litmus	Total N For period	Total N Per hr.	NH₃-N For period	NH₃-N Per hr.	Ratio
July 11, p.m.							
12.37–1.37	68	Acid	514	514	30.6	30.6	17
1.37–2.37	198	"	473	473	24.6	24.6	19
Exercise, 2.40–2.45							
2.37–3.07	22	Neutral	196	393	11.2	22.3	18
3.07–3.37	56	"	240	480	13.4	26.7	18
3.37–4.37	84	"	401	401	23.7	23.7	17

With one exception, the dogs were exercised by running at leash up and down stairs and on the level. The one exception was Dog 10, in the experiment of July 10. By this time, Dog 10 had been trained to run in a horizontal, motor-driven treadmill, and the exercise in this instance was thus accomplished. We have some reservation in interpreting the results upon Dog 11 since this dog resisted leading to the point where pressure of the harness may have impeded respiration during the exercise period.

During the time when these experiments were done the temperature ranged above 32°. In all cases, for 20 minutes or longer after exercise the dogs panted vigorously and water dripped from the tongue.

It will be noted that in two cases the preliminary diet was the low acid-forming dog biscuit ration, while in three experiments the dogs had been fed the high acid-forming diet of beef heart. At the beginning of each experiment the bladder was drained and washed with water, and water as indicated was given immediately by stomach tube. Two 1 hour control periods followed. A smaller amount of water was then given, followed immediately by exercise, and the post urine periods. Our purpose in giving water in this manner was first, to dispose of "flushing" effects within the control periods; secondly, to assure, if possible, reasonably constant and adequate urine volumes during the shorter periods following exercise. Despite this procedure, exercise invariably reduced the urine of the first after period. In three cases these reductions were to surprisingly small volumes. Thorough washing of the bladder, however, assured complete separation of all urine periods.

Little comment is required upon the results of these experiments. In those dogs which were at a relatively low level of nitrogen and ammonia excretion (upon dog biscuit diet), exercise reduces the ammonia elimination during the subsequent hour to almost negligible amounts. In the meat-fed dogs, with their high initial levels of nitrogen and ammonia excretion, the effects of exercise are not so obvious (except in Dog 11). Even in the latter group, however, if there is a significant change in rate of ammonia elimination it is in the direction of a decrease and not an increase. In four of the five experiments, the post exercise decrease in ammonia excretion is associated with a definitely decreased acidity of the urine.

We believe that the results of these experiments are in exact accord with the original theory of Nash and Benedict, and completely invalidate the strained interpretation Bliss has undertaken of associated blood changes for which we have offered in earlier sections of this paper a more rational explanation.

Blood Amide Nitrogen in Anuric Dog

If the level of blood protein amide nitrogen is a mean between synthetic processes in muscle and analytic processes in kidney, then with abolition of kidney function the level of amide nitrogen in the blood might be expected to rise as do other waste products of metabolism.

Table IX gives, we believe, a typical picture of the blood values for non-protein, protein, and protein amide nitrogen following bilateral ureteral ligation. Apparently the dog was in excellent condition until the last hour of the 5 day period following operation. The sevenfold increase in non-protein nitrogen follows a straight line curve with time. Both protein nitrogen and protein amide nitrogen show a progressive, slight fall, with no change in the percentage of amide nitrogen present in the protein molecule.

TABLE IX

Blood Protein Amide Nitrogen in Dog with Ligated Ureters

Dog 6, female; weight, 10.2 kilos. All blood samples were from the jugular vein. Diet prior to operation, Champion dog biscuit. The dog was fasted after the operation.

Time	Blood			
	Non-protein N per 100 cc.	Protein N per 100 cc.	Protein amide N	
			Per 100 cc.	Per 100 gm. protein
1931	*mg.*	*mg.*	*mg.*	*mg.*
June 3, 2.00 p.m.	30.9	2786	126.2	725
Ureters ligated 3.30 p.m.				
June 4, 9.50 a.m.	61.2	2800	124.9	714
" 5, 2.40 p.m.	113.0	2614	120.1	735
" 6, 3.25 "	149.0	2640	120.1	728
" 7, 3.15 "	176.0	2564	114.4	714
" 8, 3.45 "	216.3	2528	115.9	733

DISCUSSION

The indirect and inferential character of Bliss' study of ammonia metabolism has been criticized elsewhere (11). Recent direct studies by others (12) of the ammonia content of non-mammalian muscle have given conflicting results and sharply opposed interpretations. In our judgment, these measurements have served to emphasize the extremely labile character of postmortem formation of ammonia in such tissues. Obviously the question of *in vivo* production of ammonia by muscle is not settled.[5] The small

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[5] This is the view also of Benedict and Nash (11) to whom Bliss (5) has imputed bias for their quotation of Gad-Andresen's work (13) upon ammonia in muscle. Bliss' criticism that Benedict and Nash had ignored more recent

increments of ammonia found by Parnas, Mozołowski, and Lewiń-
ski (14) in blood from the exercised arm could have little signifi-
cance from the standpoint of neutralization of acid metabolites.
Likewise, if the reserves of ammonia or its precursor in muscle
may be exhausted by a 5 to 10 minute period of moderate running
(Bliss (3)), then the muscle tissue can hardly be thought of as
contributing any significant part of the large quantities of ammonia
which may appear in the urine over long periods of heavy acid
excretion, as in diabetes mellitus or phlorhizin poisoning.

Various other objections based on fact and theory might be
recited in rejecting the thesis supported by Bliss.   The *a priori*
difficulty in accounting for the transformation of ammonia into
blood protein amide across separating cell and blood vessel mem-
branes has been cited by Benedict and Nash (11).   Certainly
such synthesis does not occur in blood *in vitro*.   In the kidney,
likewise, it would appear that blood protein substrate and kidney
intracellular enzyme are physically separated.

Finally, it appears to us that Bliss' hypothesis violates estab-
lished facts of physiological reaction to foreign protein.   One of the
most fundamental concepts of protein structure is its specific
character.   We believe that amide groupings of the protein mole-
cule are as essential and characteristic features of its structure as
is any other part.   Replacement of carboxyl hydrogen by alkali
radical is in no way comparable to the conversion of carboxyl group
into amide structure.   Were blood proteins, or any other proteins,
capable of maintaining their identity with varying percentages of
nitrogen in their constitution, it would be necessary to revise cur-
rent notions of the mechanisms underlying protein sensitization
and allergic reaction.

BIBLIOGRAPHY

1. Bliss, S., *Science*, **67**, 515 (1928).
2. Bliss, S., *J. Biol. Chem.*, **81**, 129 (1929).
3. Bliss, S., *J. Biol. Chem.*, **81**, 137 (1929).
4. Bliss, S., *J. Biol. Chem.*, **81**, 405 (1929).

work upon this question has no basis in fact.   Benedict and Nash (11) cited
the recent work which had appeared in connection with the question of
ammonia in muscle (Parnas and Mozołowski, Embden and collaborators)
which Bliss had entirely ignored.

5. Bliss, S., *J. Pharmacol. and Exp. Therap.*, **40**, 171 (1930).
6. Mann, T., *Biochem. Z.*, **231**, 33 (1931).
7. Plimmer, R. H. A., The chemical constitution of the proteins, London and New York, 2nd edition, pt. 1, 53, 54 (1912).
8. Liljestrand, S. H., and Wilson, D. W., *J. Biol. Chem.*, **65**, 773 (1925).
9. Rice, H. A., and Steinhaus, A. H., *Am. J. Physiol.*, **96**, 529 (1931).
10. Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, **48**, 463 (1921).
11. Benedict, S. R., and Nash, T. P., Jr., *J. Biol. Chem.*, **82**, 673 (1929).
12. *Cf.* Parnas, J. K., Lewiński, W., Jaworska, J., and Umschweif, B., *Biochem. Z.*, **228**, 366 (1930); Embden, G., *Z. physiol. Chem.*, **196**, 23 (1931).
13. Gad-Andresen, K. L., *J. Biol. Chem.*, **39**, 267 (1919).
14. Parnas, J. K., Mozołowski, W., and Lewiński, W., *Biochem. Z.*, **188**, 15 (1927).

THE RING STRUCTURE OF ADENOSINE

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Previous studies[1] of inosinic acid have revealed the probability that the sugar portion of this nucleotide does not possess the pyranoside ring structure. Oxidation of the ribose phosphate from inosinic acid, by means of nitric acid, gave rise to phosphoribonic acid. Since, by this treatment, the pyranose form of ribose phosphate (whether substituted in positions (2), (3), or (4)) would give rise to a phosphoribotrihydroxyglutaric acid, it follows that position (5) of the sugar chain is protected by the phosphoryl group.

This conclusion was subsequently confirmed by an investigation[2] of the rate of lactone formation. The transformation of the phosphoribonic acid to its lactone was found to proceed very slowly, a property characteristic of <1,4> lactones. The formation of <1,5> lactone was not observed, as should be the case when position (5) of the sugar is substituted.

As no cyclic sugar derivatives are yet known in which the ring structure does not conform either to the pyranoside or else the furanoside type, it seemed warranted to attribute to inosinic acid the structure of hypoxanthine 5-phosphoribofuranoside.

Now, assuming that dephosphorylation involves no change in ring structure (the reducing group remaining protected by the purine base residue), it would follow that inosine also possesses the <1, 4> lactal structure and since adenosine is readily transformed to inosine[3] having properties identical with those of the inosine

[1] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **44**, 746 (1911).

[2] Levene, P. A., and Mori, T., *J. Biol. Chem.*, **81**, 215 (1929).

[3] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **43**, 3161 (1910).

from inosinic acid, it was to be expected that adenosine likewise possesses the furanoside ring structure.

These conclusions, however, eliminate the suggestions of Robinson[4] who upholds the view that ribose, as such, does not exist in the molecule of plant nucleic acid or in the nucleotides derived from it, but is a "reversion" product formed by a Walden inversion during the dephosphorylation of some other pentose phosphate derivative. He suggests that d-xylose is this pentose and his hypothesis requires that the phosphoric acid residue be attached to some position of the sugar chain *other than position (5)*.

The arguments used by this author have no experimental justification and reasons have already been given[5] for doubting their validity. The question has now been put to a further experimental test by the preparation, from adenosine, of ribose derivatives which indubitably have the furanose structure. These new substances show no relationship to the corresponding ones from normal methylriboside but are, on the other hand, typical γ-sugar derivatives.

Adenosine was completely methylated by taking advantage of the solubility of adenosine acetate in acetone. The addition of dimethyl sulfate and alkali to such a solution introduced in one operation the theoretical percentage of methoxyl, whereas previous attempts[6] at methylation of adenosine and related substances (by more direct methods) had failed owing to their ease of hydrolysis and ready destructibility. The product, trimethyl N-methyladenosine, was isolated in beautifully crystalline condition, in the form of its hydrochloride.

Hydrolysis of this completely methylated nucleoside gave crystalline monomethyladenine and a new sugar, the composition of which corresponded to that for trimethyl ribose.

The general properties of this sugar differentiated it sharply from 2, 3, 4-trimethyl ribose;[7] in particular, its physical condition at room temperature, its specific rotation, and its ease of condensation at room temperature with methyl alcohol containing hydrogen chloride.

[4] Robinson, R., *Nature*, **120,** 44, 656 (1927).

[5] Levene, P. A., *Nature*, **120,** 621 (1927).

[6] Levene, P. A., *J. Biol. Chem.*, **55,** 437 (1923). Case, F. H., and Hill, A. J., *J. Am. Chem. Soc.*, **52,** 1536 (1930).

[7] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93,** 623 (1931).

```
N=C—NH₂                              N=C—NH · CH₃ · HCl
  |   |                                |   |
H · C  C————————N                    H · C  C————————N
  |   /                                |
N—C—N=CH  |                          N-      -N=CH
          |                               —
          |
        H · C————————                    H · C-
          |                  |
        H · C · OH            |           H · C · OMe
          |                   O                        O
        H · C · OH            |           H · C · OMe
          |                   |
        H · C————————                    H · C-
          |
        CH₂OH                             CH₂OMe
```

Adenosine   Trimethyl N-methyladenosine hydrochloride

```
  CHOH————————┐              CO————————┐              COOH
    |         |                |       |                |
  H · C · OMe |              H · C · OMe |            H · C · OMe
    |         O                |        O               |
  H · C · OMe |              H · C · OMe |            H · C · OMe
    |         |                |       |                |
  H · C————————┘              H · C————┘              COOH
    |                          |
  CH₂OMe                       CH₂OMe
```

2,3,5-Trimethyl ribose   Trimethyl γ-ribono-   i-Dimethoxysuccinic
                                  lactone   acid
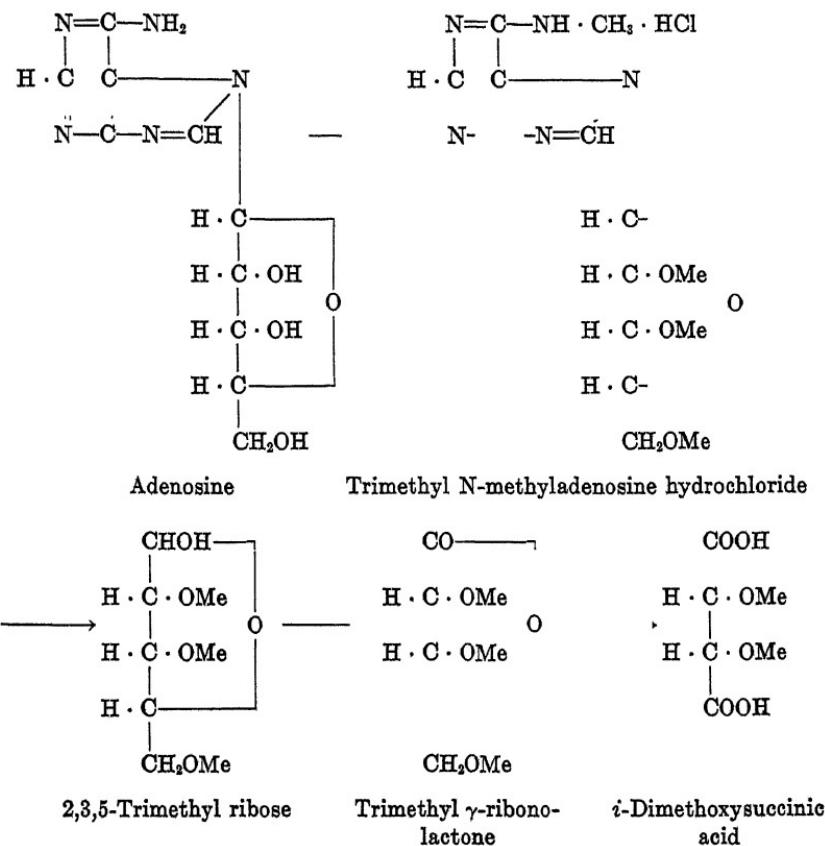
Evidence of the presence of a furanose ring structure in this sugar was provided by oxidizing the substance with nitric acid under conditions in which trimethyl ribopyranose gives trimethoxyglutaric acid exclusively. In the present instance the sole product was i-dimethoxysuccinic acid, which was isolated and identified in the form of its crystalline dimethyl ester.

Confirmatory evidence concerning the structure of the sugar was obtained from the nature of the trimethyl ribonolactone derived from it by the action of bromine water. The lactone displayed the behavior typical of a γ-lactone, since polarimetric observations during its hydrolysis in aqueous solution showed that the conversion of lactone into acid proceeded so slowly that after 500 hours equilibrium was far from being attained. It is similar in its rate

of hydrolysis to tetramethyl γ-mannonolactone and trimethy γ-lyxonolactone to which it is related in the configuration of carbon atoms (2) and (3). In addition, this lactone provides still a further example of the profound influence[7,8] exerted by the solvent upon the specific rotation of methylated sugar lactones having *cis* methoxyl groups attached to carbon atoms (2) and (3).

<div align="center">EXPERIMENTAL</div>

Preparation of Adenosine Acetate—A suspension of 2 gm. of fused sodium acetate in 130 cc. of acetic anhydride was heated to gentle ebullition in a wide necked Erlenmeyer flask over a wire gauze. Then adenosine hydrate (10 gm.) was added in portions sufficient to keep the mixture boiling. When all the adenosine had been added the resultant pale yellow solution was poured, while hot, into 500 cc. of cold toluene, the solution cooled in ice to room temperature, and the sodium acetate which separated was filtered off and washed at the pump with toluene. The combined filtrate was evaporated to a thick gum under diminished pressure at 40°. Two further portions of toluene were run in and evaporated off. The resulting thick, pale yellow gum was dissolved in 250 cc. of acetone, the traces of undissolved sodium acetate filtered off, and the filtrate evaporated to dryness under diminished pressure. The adenosine acetate was obtained as a pale yellow, flaky glass-like solid. Yield, 13 to 15 gm. The product was insoluble in cold or hot petroleum ether; insoluble in cold but somewhat soluble in hot ether; and quite soluble in the following solvents in the cold: chloroform, glacial acetic acid, pyridine, acetone, methyl alcohol, ethyl alcohol, ethyl acetate, and water. It gradually dissolved in warm 30 per cent sodium hydroxide solution to give a colorless solution but on continued heating this became deep yellow, then darker yellow, and finally greenish brown in color.

Preparation of Fully Methylated Adenosine in Two Operations— The following method, used in preliminary experiments, gives in two operations a methylated adenosine with a methoxyl content of 25.3 per cent (for the hydrochloride). It consists essentially in the simultaneous deacetylation and methylation of adenosine acetate dissolved in acetone in a large flask provided with a reflux

[8] Haworth, W. N., Hirst, E. L., and Smith, J. A. B., *J. Chem. Soc.*, 2659 (1930).

condenser and an efficient mechanical stirrer.   The methoxyl content of the product may be varied by altering the proportion of acetone employed and in this way methylated adenosines containing 16 per cent to 20 per cent methoxyl (in the hydrochloride)ʹ have been obtained in one treatment, but after one further treatment, a fully methylated product results.

19 gm. of adenosine hydrate were acetylated, the product dissolved in 76 cc. of acetone, and treated with 50 cc. of dimethyl sulfate and 110 cc. of 30 per cent aqueous sodium hydroxide at 50–70° during 50 minutes, the temperature being finally maintained at 70° for a further 30 minutes during which period the color changed from light to dark brown.   The acetone was now removed under diminished pressure at 60°, the solution was cooled to 0° and made almost neutral with 10 per cent sulfuric acid, the neutralization being completed by passing in carbon dioxide.   The solution was extracted repeatedly with chloroform and then the aqueous layer was evaporated to dryness and the solid product extracted with boiling chloroform under a reflux.   The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under diminished pressure.   The product was dissolved in absolute methyl alcohol and again evaporated to dryness, giving 19.5 gm. of brown gum.   This was dissolved in a mixture of 70 cc. of absolute methyl alcohol and 30 cc. of dry ether, cooled to 0°, and dry hydrogen chloride was passed in to slight excess.   After being kept in the refrigerator for several hours the mixture was filtered and the crystalline material dried. Yield, 17 gm.

5.572 mg. substance: 7.475 mg. AgI
$C_{14}H_{22}O_4N_5Cl$.   Calculated.   MeO 25.87.   Found.   MeO 17.72

6 gm. of the product were made faintly alkaline by addition of 0.5 N sodium hydroxide solution from a burette and the solution was treated with 15 cc. of dimethyl sulfate and 29 cc. of 30 per cent sodium hydroxide during 30 minutes, the temperature being raised from 50 to 60°.   The solution became opalescent and an oily substance separated on the surface.   The temperature was then maintained at 70° during a further 150 minutes, the color of the solution remaining pale yellow.   The product was isolated in

the form of the hydrochloride. Yield, 5 gm. It had the following composition.

.   5.000 mg. substance: 8.550 mg. CO$_2$ and 2.620 mg. H$_2$O
    4.390 "   "   : 1.683 "   AgCl
    5.137 "   "   : 0.877 cc. N$_2$ (759 mm. at 28°)
    5.098 "   "   : 9.769 mg. AgI
    C$_{14}$H$_{22}$O$_4$N$_5$Cl.
    Calculated.   C 46 71, H 6.2, N 19.47, Cl 9.86, MeO 25.87, amino N 0.00
    Found.   " 46.64, " 5.9, " 19.32, " 9.48,   " 25.32,   "   " 0.00
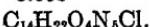
The following method gives, in one operation, a methylated adenosine with a methoxyl content of 25.6 per cent (in the hydrochloride). The method differs from that described above in the proportion of acetone and methylating agents employed, the lower temperature maintained during the methylation, and the greater length of time allowed for completion of the reaction.

Preparation of Fully Methylated Adenosine in One Operation— 15 gm. of flaky adenosine acetate were dissolved in 300 cc. of acetone and treated with 249 cc. of 30 per cent sodium hydroxide solution and 122 cc. of dimethyl sulfate. The methylating reagents were added, dropwise (with the usual precautions) in twenty equal portions at intervals of 5 minutes, the temperature being maintained at 55°. After the addition of the reagents the solution was heated for 1 hour at 60°, the resulting solution being only very pale yellow in color. Two layers had then formed, a gummy white solid separating the two. The acetone was now removed under diminished pressure at 40° and the white powdery substance was filtered off, dried, and extracted several times with boiling chloroform under a reflux. The aqueous filtrate was cooled to room temperature and extracted repeatedly with chloroform. The united chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to a yellow gum which was dissolved in methyl alcohol and the solution reevaporated to remove traces of chloroform. The product was a yellow, glassy solid, soluble in acetone, methyl alcohol, and water; insoluble in ether or petroleum ether. Yield, 10.5 gm. It was dissolved in 35 cc. of absolute methyl alcohol, 15 cc. of dry ether were added, and the solution was cooled to 0° in a freezing mixture. A slow stream of dry hydrogen chloride was now passed into the solution, with shaking, the temperature being maintained at 0° and after the

addition of slightly more than the theoretical amount of hydrogen chloride a solid mass of crystals had separated. The mixture was kept in the refrigerator for several hours and then filtered and dried in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide. Yield, 6.5 gm. of colorless crystals.

The substance had the following composition.

4.785 mg. substance: 0.817 cc. N_2 (753.5 mm. at 25°)
4.772 "   "   : 9.250 mg. AgI
Amino nitrogen: 0.00
    $C_{14}H_{22}O_4N_5Cl$. Calculated. N 19.47, MeO 25.87, amino N 0.00
                  Found.   " 19.39, " 25.61,   "   " 0.00

The hydrochloride crystallizes in rosettes of colorless fine needles from absolute methyl alcohol containing a trace of hydrogen chloride. After hydrolysis by boiling with hydrochloric acid, it reduces Fehling's solution in the manner characteristic of a mixture of sugar and purine base. It is soluble in methyl alcohol, chloroform, and water but insoluble in dry ether and decomposes at 210° without melting. It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.44° \times 100}{2 \times 1.040} = -21.2° \text{ (in water)}$$

Hydrolysis of Trimethyl N-Methyladenosine—6 gm. of the crystalline hydrochloride were dissolved in 120 cc. of 4 per cent hydrochloric acid and the resultant solution was heated under a reflux condenser in a bath at 85° for 2 hours. The hot solution was then made neutral by addition of barium carbonate, heated till boiling to expel excess carbon dioxide, and then kept in the refrigerator overnight. The base which had separated was filtered off and washed well with chloroform and was then dissolved in 50 cc. of boiling water. The small amount of barium carbonate was filtered off, the solution heated with a little charcoal, filtered, and cooled to 0°. The base, which crystallized in matted, fine, silky needles, was filtered off and dried. It did not melt at 275°⁹ and had the following composition.

5.196 mg. substance: 9.161 mg. CO_2 and 2.258 mg. H_2O
2.989 "   "   : 1.237 cc. N_2 (29°, 765 mm.)
Amino N: 0.00
    $C_6H_7N_5$. Calculated. C 48.29, H 4.7, N 46.98, amino N 0.00
              Found.   " 48.08, " 4.9, " 47.07,   "   " 0.00

The aqueous solution was extracted several times with chloroform and then evaporated to dryness and the barium chloride extracted with boiling chloroform under a reflux. The united chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness under diminished pressure. The resulting syrup was dissolved in ether, a small amount of flocculent material filtered off, and the filtrate evaporated under diminished pressure to a mobile syrup (2.5 gm.) which was distilled at high vacuum.

A colorless mobile liquid was collected at 90–92° at 0.02 mm. (bath temperature, 116°). Yield, 2.0 gm. $N_D^{26} = 1.4531$. It was strongly reducing towards boiling Fehling's solution and had a bitter taste.

It had the following composition.

4.085 mg. substance: 7.540 mg. CO_2 and 3.136 mg. H_2O
5.306 " " : 19.230 " AgI
$C_8H_{16}O_5$. Calculated. C 49.97, H 8.4, MeO 48.45
Found. " 50.33, " 8.6, " 47.88

The syrupy distilled product had the following specific rotations.

$$[\alpha]_D^{26} = \frac{+ 1.46° \times 100}{2 \times 1.414} = + 51.6° \text{ (in water)}$$

$$[\alpha]_D^{26} = \frac{+ 1.13° \times 100}{2 \times 1.222} = + 46.2° \text{ (in absolute methyl alcohol)}$$

It condensed rapidly at 27° with methyl alcohol containing 1 per cent of hydrogen chloride. The results obtained when $l = 2$ dm., and $c = 1.2$ are given in Table I. Under similar conditions the condensation of crystalline trimethyl ribopyranose with methyl alcohol was considerably different. For purposes of comparison a specimen of crystalline 2, 3, 4-trimethyl ribose was distilled at high vacuum. It distilled at 85–90° at 0.05 mm. (bath temperature, 115–120°) and in the superfused state had $N_D^{25} = 1.4550$. It crystallized again on nucleation, and the crystalline material had the following specific rotation.

$$[\alpha]_D^{26} = \frac{- 0.60° \times 100}{2 \times 1.124} = - 26.7° \text{ (in absolute methyl alcohol)}$$

Its behavior when dissolved at 26° in methyl alcohol containing 1 per cent of hydrogen chloride was studied polarimetrically. The results obtained when $l = 2$ dm., and $c = 1.1$ are given in Table II.

Oxidation of Trimethyl Ribofuranose—1.6 gm. of trimethyl ribose were dissolved in 20 cc. of water and 2 cc. of bromine were added in portions of 0.5 cc., during 4 days at 38°. The excess bromine was then removed by aeration, silver oxide was added until all the

TABLE I
Methylglycoside Formation at Room Temperature

Time	$[\alpha]_D^{27}$	Time	$[\alpha]_D^{27}$
min.		*min.*	
2	+40.5	25	+15.0
4	+36.7	26	+13.8
6	+30.5	65	+15.4
7	+29.2	85	+15.4
10	+23.8	340	+18.8
15	+18.4	1780	+18.8
20	+15.4		

TABLE II
Methylglycoside Formation of 2, 3, 4-Trimethyl Ribose at Room Temperature

Time	$[\alpha]_D^{26}$
hrs.	
0.5	+24.8
1.0	+23.0
6.0	+14.8
18.5	+6.4
28.0	+2.2
44.0	−1.8
67.0	−4.4

mineral acid had been neutralized, the mixture was filtered, and the silver salts well washed with hot water. To the combined aqueous solution was added dilute hydrochloric acid from a burette until all the silver in solution had been precipitated. The mixture was filtered and the filtrate evaporated to a thick syrup under diminished pressure. The product was heated at 100° at 15 mm. for 5 hours in order to complete lactonization and then distilled at high vacuum. The main fraction (weight, 1.3 gm.) boiled at 110–

115° at 0.2 mm. (bath temperature, 130–135°). It was a colorless, quite mobile liquid and had $N_D^{26.5} = 1.4501$. It had the following composition.

5.748 mg. substance: 10.603 mg. CO_2 and 3.820 mg. H_2O
4.820 "   "   : 17.910 " AgI
        $C_8H_{14}O_5$. Calculated. C 50.50, H 7.4, MeO 48.96
               Found.   " 50.31, " 7.4, "   49.06
100 mg. substance required: 5.34 cc. 0.1 N NaOH
$C_8H_{14}O_5$.   Calculated. 5.26 " 0.1 "   "

The substance displayed the following specific rotations.

$$[\alpha]_D^{24} = \frac{+1.06° \times 100}{2 \times 0.948} = +55.9° \text{ (in chloroform)}$$

$$[\alpha]_D^{24} = \frac{+2.38° \times 100}{2 \times 0.960} = +124.0° \text{ (in dry ether)}$$

$$[\alpha]_D^{24} = \frac{+2.28° \times 100}{2 \times 1.372} = +83.1° \text{ (in benzene)}$$

The course of the hydrolysis of the lactone in aqueous solution was studied polarimetrically. The results obtained when $l = 2$ dm., and $c = 1.3$ are given in Table III.

The rotation of the free acid was determined in the usual manner by forming the sodium salt, adding the equivalent amount of hydrochloric acid, and observing the rotation immediately. Calculated as lactone, the initial specific rotation was as follows:

$$[\alpha]_D^{24} = \frac{+0.55° \times 100}{2 \times 0.674} = +40.8° \text{ (in water)}$$

After 448 hours the specific rotation was

$$[\alpha]_D^{24} = \frac{+0.16° \times 100}{2 \times 0.674} = +11.9° \text{ (in water)}$$

Oxidation of Trimethyl Ribofuranose with Nitric Acid—1.5 gm. of syrupy distilled trimethyl ribose were dissolved in 15 cc. of concentrated nitric acid (sp. gr., 1.42) at 26° and the solution was kept at room temperature overnight. It was now warmed in a bath at 95° during 7 hours after which gas evolution had ceased. The reaction mixture was cooled, diluted with distilled water, and evaporated

under diminished pressure at 45° with continual additions of water. This procedure was repeated many times until the final distillate was free from nitric acid (2 days). The product was an entirely crystalline mass. It was dried by dissolving in absolute methyl alcohol and again evaporating to dryness, after which it was esterified by boiling for 6 hours with 50 cc. of methyl alcohol containing 2 per cent of hydrogen chloride. After the mixture had been cooled, and the mineral acid neutralized with dry silver carbonate, the methyl alcohol was removed by evaporation under diminished pressure giving a completely crystalline product which was purified by distillation at high vacuum. A colorless syrupy product, which crystallized immediately in the receiver, was col-

TABLE III

Conversion of Lactone into Acid

Time	$[\alpha]_D^{27}$	Time	$[\alpha]_D^{27}$
hrs.		*hrs.*	
0	−18.9	394	+1.5
56	−15.1	466	+4.5
244	−4.5	514	+5.3
292	−2.3	703	+7.6
342	−0.8		

lected at 80° at 0.2 mm. (bath temperature, 90–95°). Yield, 0.79 gm.; melting point crude, 66–67°. It was recrystallized from ether-light petroleum ether, being obtained in flat plates (m.p., 68° alone or admixed with an authentic specimen of dimethyl *i*-dimethoxysuccinate prepared from mesotartaric acid.)

It had the following composition.

4.837 mg. substance: 8.304 mg. CO_2 and 2.846 mg. H_2O
3.726 "   "   : 16.910 " AgI
       $C_8H_{14}O_6$. Calculated. C 46.58, H 6.8, MeO 60.21
              Found.   " 46.81, " 6.6, "   59.96

It was optically inactive under conditions ($c = 1.0; l = 2$ dm.) where a specific rotation of ± 1° would easily have been detectable. The melting point, optical inactivity, and analytical figures showed it to be the dimethyl ester of inactive dimethoxysuccinic acid.

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